# REMARKS

After amendment, claims 9-10, 12-15 and 19-21 are pending in the present application. The amendment to the claims has been made to address the Examiner's §112, second paragraph rejection by making the claims more definite. Support for the amendment to the claims can be found in the specification, including the originally filed claims. The claims as drafted are narrower versions of the claims which were on file as of the presentation of the preliminary amendment of December 5, 2001.

The Examiner has rejected claims 9-15 and 19-21 variously under 35 U.S.C. §112, first and second paragraphs. Applicants shall address each of the Examiner's rejections in the accompanying sections of this paper.

## The 35 U.S.C. §112, First Paragraph Rejection

The Examiner has rejected claim 9-15 and 19-21 as containing subject matter which was not described in the specification in such as way as to enable one of ordinary skill in the art to which it pertains to make and/or use the invention. In particular, the Examiner contends that the teachings of the specification do not provide sufficient basis upon which to enable the claimed inventions. The specific reasons are set forth on pages 2-7 of the office action. For the reasons which are set forth hereinbelow, Applicants respectfully traverse the Examiner's rejection.

The present invention which is set forth in claims 9-10, 12-15 and 19-21 is directed to arsenic-substituted oxazole compounds, a method for inhibiting cell surface protein disulfide isomerase, a method for treating a patient for a viral infection propagated by PDI-mediated virion entry into host cells and a method for determining optimum blood concentrations of the arsenic-substituted oxazole PDI inhibitor compounds.

The Examiner contends that the present invention is non-enabled. Applicants respectfully traverse the Examiner's rejection. Inasmuch as claims 9-10 are directed to certain arsenic-substituted oxazole compounds according to the very definite structure set forth in claim 9 and defined therein in claims 9 and 10, it is respectfully posited that claim 9-10 are enabled. The specification provides a clear method for providing compounds according to claim 9-10 generally, in the specification and more specifically in the specification at pages 24-32. These compounds may be prepared accordingly by following the disclosed methodology by analogy. Use of the compounds is readily accomplished according to the specification by following the teachings throughout the specification and in particular, at page 13-14. The compounds may be used to inhibit cell surface PDI and also, may be administered orally or parenterally to a patient, as indicated, in effective amounts. A method for determining optimum blood concentrations of a PDI inhibitor is disclosed in great detail on pages 13-14 of the present application.

In all instances, the claimed invention is described in sufficient detail to allow one of ordinary skill to make and use the invention without engaging in undue experimentation. Each aspect of the claimed invention is described in the specification and one of ordinary skill can make and use the invention either following the teachings directly or by analogy using methods which are well within the skill of the routineer. That is all that is required by 35 U.S.C. §112, first paragraph. That is not to say that no additional experimentation will need to be conducted in order for the person of ordinary skill to make and use the invention. That is to say, however, that whatever additional experimentation may be needed will not be *undue* experimentation.

The Examiner appears to posit that the presently claimed compounds and methods are not predictable from the specific teachings of the specification and because of that absence of predictability, the Examiner rejects the present claims. Applicants respectfully traverse the Examiner's rejection. Although the various compounds and methods will act with some

measure of variation, it is respectfully submitted that there is predictability in that the inventions are expected to exhibit activity and to work at least to some degree. Note that the embodiments which are embraced by one or more claims do not have to be predictive of commercial success, only that the compounds have an expectation of being useful within the context of the claimed invention. In support of such a proposition, Applicants provide copies of several references, which support the general expectation that the compounds according to the present invention are useful for the claimed purposes, would inhibit PDI (which is involved in HIV infection) and would be useful in the treatment of HIV infections in patients. See, for example, the enclosed copies of Bennett, et al., Sulhydryl Regulation of L-Selectin Shedding: Phenylarsine Oxide Promotes Activation-Independent L-Selectin Shedding from Leukocytes, *Journal of Immunology*, 164, 4120 (2000); Matthis, et al., Disulfide exchange in domain 2 of CD4 is required for entry of HIV-1, *Nature Immunology*, vol. 3, no. 8, pp. 727-732 (August, 2002); Goldsmith and Doms, HIV entry: are all receptors created equal?, *Nature Immunology*, vol. 3, no. 8, pp. 709-710 (August, 2002); and Barbouche, et al., Protein-disulfide Isomerase-mediated Reduction of Two Disulfide Bonds of HIV Envelope Glycoprotein 120 Occurs Post-CXCR4 Binding and Is Required for Fusion, *Journal of Biological Chemistry*, Vol. 278, No. 5, pp. 3131-3136 (January 31, 2003) in support of the patentability of the presently claimed invention.

It is respectfully submitted that the teachings of the specification meet the requirements of 35 U.S.C. §112, first paragraph. It is noted here that claimed compounds represent oxazole compounds which are substituted with an As=O group at the 4-position of the the oxazole moiety and an R' group at the 3-position and an R group at the 5-position of the oxazole moiety. In the present invention, at least one of R or R' is a charged ligand, preferably a sulphonate group, and the other of R or R' is optionally H or a $C_1$-$C_6$ alkyl group. Because the enzyme which is inhibited by the presently claimed compounds, PDI, is a highly flexible chaperone enzyme involved in carrying proteins and other complex molecules (including viruses) into the cell, it is a broadly accommodative enzyme (as taught in the specification) with two cysteinyl residues at its

active site and virtually no steric structural impediments to binding with compounds of the present invention. The enzyme can accommodate macromolecules, including a number of polypeptides. The As=O group of the present compounds is known to bind tightly to the mercapto groups of cysteinyl residues and charged molecules at physiological pH are known to have difficulty pentrating cells. See, Bennett, et al., supra. Taken together, the compounds of the present invention, which have few steric limitations are expected to have no trouble reaching the active site of PDI, where the As=O moiety binds to at least one of the cysteinyl residues in the active site of that enzyme. The charged ligand prevents the compound's penetration of the cell.

With respect to the claims directed to the inhibition of PDI, the basic chemical structure of the claim 9 compounds raises the expectation that these compounds function as good inhibitors of PDI, especially given the general reactivity of the As=O moiety with the cysteinyl groups which are at the active site of the PDI enzyme.

It is also respectfully submitted that the claims directed to the method of treating a mammal for a viral infection propagated by PDI-mediated virion entry into host cells is respectfully enabled by virtue of the expectation of PDI inhibition by the presently claimed compounds. PDI inhibition results in the inability of a virus to propagate through a PDI-mediated mechanism and consequently the virus in the mammal is treated accordingly. This is clearly taught by the present invention. Determining an effective ("sufficient") amount of compound to provide such a result by following the teachings of the specification and in particular, the teachings on pages 13-14 of the present specification.

Regarding the enablement of claims 19-21, it is respectfully submitted that the teachings of the specification are sufficient to provide enablement for practicing the invention. Following the teachings in the specification at pages 13-20, and simply providing results of the testing will allow one of ordinary skill to determine the lowest concentration of inhibitor which will provide

optimum activity, resulting in a determination of an optimum blood concentration of inhibitor. This is a relatively facile undertaking. Thus, one of ordinary skill following the teachings of the specification can readily practice the invention of claims 19-21.

It is therefore respectfully submitted that the present invention, as set forth in pending claims 9-10, 12-15 and 19-21, are in compliance with the requirements of 35 U.S.C. §112, first paragraph.

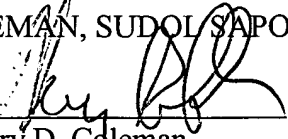The 35 U.S.C. §112, Second Paragraph Rejection

The Examiner has rejected claims previously submitted claims 9-15 and 19-21 under 35 U.S.C. §112, second paragraph for the reasons which are stated in the office action on pages 7-8. In response, Applicants have amended the claims to address the Examiner's rejections. Claims 9, 12, 13, and 19 have been amended. It is respectfully submitted that with the amendment to the claims, the application is in compliance with the requirements of 35 U.S.C. §112, second paragraph. Note that claim 19 contains active steps- an admixing step and an assaying step and is believed to meet the requirements of 35 U.S.C. §112, second paragraph.

For the above reasons, Applicants respectfully assert that the claims set forth in the amendment to the application of the present invention are now in compliance with 35 U.S.C. Applicants respectfully submit that the present application is now in condition for allowance and such action is earnestly solicited. Applicants have cancelled several dependent claims and added no new claims. No fee is therefore due for the presentation of this amendment. If any additional fee is due or any overpayment has been made, please charge/credit Deposit Account No. 04-0838.

The Examiner is cordially requested to call the undersigned attorney if the Examiner believes that a telephonic discussion may materially advance the prosecution of the instant application in any way. Copies of cited references are enclosed for the Examiner's review.
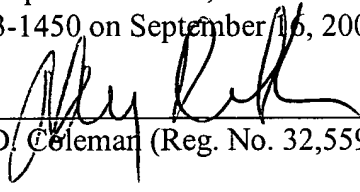
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# Sulfhydryl Regulation of L-Selectin Shedding: Phenylarsine Oxide Promotes Activation-Independent L-Selectin Shedding from Leukocytes[1]

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The L-selectin adhesion molecule mediates leukocyte recruitment to inflammatory sites and lymphocyte trafficking through the peripheral lymph nodes. In response to leukocyte activation, L-selectin is proteolytically released from the cell surface, disabling leukocytes from the subsequent L-selectin-dependent interactions. We have found that L-selectin shedding is sensitive to sulfhydryl chemistry; it is promoted by thiol-oxidizing or -blocking reagents and inhibited by reducing reagents. Phenylarsine oxide (PAO), a trivalent arsenical that interacts with vicinal dithiols, is most potent in inducing rapid shedding of L-selectin from isolated neutrophils, eosinophils, and lymphocytes as well as from neutrophils in whole blood. PAO does not cause cell activation, nor does it interfere with integrin function or alter the expression of several other cell surface molecules at the low concentrations that induce L-selectin shedding. PAO is not required to enter the cell to induce L-selectin shedding. TAPI-2 ((N-{D,L-[2-(hydroxyaminocarbonyl)-methyl]-4-methylpentanoyl}-L-3-(tert-butyl)-alanyl-L-alanine, 2-aminoethyl amide), which has previously been shown to inhibit the activation-dependent L-selectin shedding, is also capable of inhibiting PAO-induced L-selectin shedding. We hypothesize that PAO-induced L-selectin shedding involves a regulatory molecule, such as protein disulfide isomerase (PDI), an enzyme that plays a role in the formation and rearrangement of disulfide bonds, contains PAO-binding, vicinal dithiol-active sites, and is expressed on the neutrophil surface. Cell surface expression of PDI, L-selectin shedding induced by PDI-blocking Abs and by bacitracin, a known inhibitor of PDI activity, and direct binding of PDI to PAO, provide supporting evidence for this hypothesis. *The Journal of Immunology*, 2000, 164: 4120–4129.

T he recruitment of neutrophils to inflammatory sites and lymphocyte trafficking among blood, lymphoid, and non-lymphoid tissues involve a regulated sequence of adhesive interactions among adhesion molecules of the selectin, mucin, integrin, and ICAM families (1, 2). At the receptor level, expression, conformation, and proteolytic cleavage are among the mechanisms by which cellular adhesive interactions can be controlled. L-selectin is expressed on the majority of leukocytes, including peripheral blood T and B lymphocytes, neutrophils, eosinophils, basophils, monocytes, NK cells, and some subpopulations of thymocytes (3). Under flow-induced high shear forces, L-selectin plays a critical role in initiating the interactions of these cells with the activated endothelium at an inflammatory site (4, 5). In addition, L-selectin is thought to amplify the inflammatory process by permitting adherent neutrophils to recruit additional neutrophils (6–8). Lymphocytes require L-selectin for trafficking across the high endothelial venules into the peripheral lymph nodes (2) and to discriminate between the Th1- vs. Th2-type cytokine-producing T cells (9). L-selectin also plays a decisive role in the development

of other neutrophil- and lymphocyte-mediated pathological processes, including ischemia-reperfusion injury, septic shock, graft rejection, autoimmune diseases, the metastasis of lymphoid tumors (10–13), and HIV-induced CD4$^+$ cell depletion (14).

L-selectin shedding is one important aspect of the normal physiologic regulation of L-selectin adhesive function. Cell surface expression of this adhesion molecule is characteristically down-modulated in response to cell activation (3, 15, 16). This proteolytic release from neutrophils inhibits subsequent L-selectin-dependent interactions with other neutrophils and endothelial cells at inflammatory sites (17, 18). Lymphocyte L-selectin is shed in response to activation by PMA (15), bacterial superantigens (19), or, like neutrophil L-selectin, by the treatment of cells with Abs to L-selectin (20, 21). L-selectin loss results in profound changes in T cell recirculation pathways (22), and studies with L-selectin-deficient mice have revealed a dramatic (70–90%) reduction in the number of lymphocytes in peripheral lymph nodes (22, 23). The released, soluble L-selectin retains binding capacity and may function as an adhesive buffer by preventing leukocyte adhesion at sites of subacute inflammation (24). Increased levels of plasma L-selectin are found in several disease states, including AIDS (25).

L-selectin shedding is the result of a proteolytic cleavage close to its transmembrane domain, conducted by a constitutively active membrane metalloprotease (26, 27), recently shown to be identical with TNF-α converting enzyme (28). Several groups, including ours, have shown that hydroxamic acid-based inhibitors of matrix metalloproteases, such as (N-{D,L-[2-(hydroxyaminocarbonyl)-methyl]-4-methylpentanoyl}-L-3-(tert-butyl)-alanyl-L-alanine,2-aminoethyl amide (TAPI-2),[3] inhibit the L-selectin sheddase and

have used these compounds to study the physiological consequences of L-selectin retention (18, 29–31). While it is clear that the susceptibility to this protease is determined by the tertiary structure of L-selectin, the mechanism by which these conformational changes are modulated remains unclear (27, 32). Interestingly, calmodulin has recently been found to be associated with the cytoplasmic domain of L-selectin, and calmodulin inhibitors were shown to induce L-selectin shedding through a protease-dependent mechanism (33).

We report here on the regulation of L-selectin shedding by sulfhydryl reagents. In an effort to begin to understand the mechanisms of shedding, we have studied in detail the effect of phenylarsine oxide (PAO), which we found induces activation-independent L-selectin release from neutrophils, lymphocytes, and eosinophils. PAO is an organic trivalent arsenical that cross-links vicinal thiols in the Cys-x-y-Cys sequence by forming stable dithioarsine rings (34, 35). The dithiols 2,3-dimercaptopropanol (DMP), also known as British anti-lewisite, and its membrane-impermeable sulfonic acid analogue 2,3-dimercaptopropanesulfonic acid (DMPS), known to remove PAO from its protein target(s) (36), effectively block PAO-induced L-selectin shedding. PAO affects many cell functions, including receptor internalization (37), glucose uptake (38), neutrophil NADPH oxidase (39), platelet activation (40), protein tyrosine phosphatase activity (41), and IL-1 converting enzyme-related apoptosis (42). Although most of these effects are imparted at low concentrations of PAO ($\ll$10 $\mu$M), they may require PAO to enter the cell. Here we present evidence suggesting that PAO induces L-selectin shedding by interacting with a cell surface target and that the entrance of PAO into the cell is not required. Moreover, we propose that a likely target of PAO in this process is a membrane-resident protein disulfide isomerase (PDI) (43–45), a redox-sensitive enzyme that catalyzes oxidation-reduction reactions through an internal, vicinal dithiol-dependent, disulfide-sulfhydryl interchange.

## Materials and Methods

### Neutrophil, lymphocyte, and eosinophil isolation

Human venous blood was collected from healthy volunteers into sterile syringes containing heparin (10 U/ml of blood; Elkins-Simms, Cherry Hill, NJ). The blood was separated on Mono-Poly resolving medium (ICN Biochemicals, Aurora, OH) by centrifugation at 500 $\times$ $g$ for 22 min at 12°C. The granulocyte and mononuclear (for lymphocytes) layers were collected separately and washed in HHB buffer (110 mM NaCl, 10 mM KCl, 10 nM glucose, 1 mM MgCl$_2$, and 30 mM HEPES, pH 7.40), then pelleted at 400 $\times$ $g$ for 10 min. The cells were resuspended in HHB buffer containing 0.1% human serum albumin (Armour, Kankakee, IL) and 1.5 mM CaCl$_2$, at 10$^7$ cells/ml. The buffer was depleted of endotoxin by affinity chromatography over columns containing polymyxin B-Sepharose (Detoxi-gel, Pierce, Rockford, IL) and autoclaving for 1 h. All plastic ware was autoclaved for at least 45 min. Eosinophils were identified by labeling the granulocyte population with VLA-4 mAb (IgG1, PE-anti human CD49d, PharMingen, San Diego, CA) at 0.50 $\mu$g/ml, then gating on the FL2-positive population with a FACScan cytometer (Becton Dickinson, Lincoln Park, NJ). This method of identifying eosinophils was verified by flow cytometry cell sorting (Elite, Coulter, Miami, FL) of very late Ag-4 (CD49d) and L-selectin-positive granulocytes and subsequent immunohistochemical analysis of the sorted population (Ref. 46 and E. B. Lynam and L. A. Sklar, unpublished observations).

### Reagents

Neutrophils were activated with fMLF (Sigma, St. Louis, MO) for 10 min at 37°C at a final concentration of 100 nM. PAO, DMP, DMPS, $N$-ethylmaleimide, $N$-acetyl-L-cysteine, glutathione, iodoacetate, nitro blue tetrazolium, iodoacetamide, mersalyl acid ($o$-((3-hydroxomercurio-2-me-

thoxypropyl)carbomyl)phenoxyacetic acid), thimerosal (mercury-(($O$-carboxyphenyl)thio)ethyl sodium salt), 5,5'-dithio-bis-(2-nitrobenozoic acid) (DTNB), PMSF, and $p$-aminophenylmercuric acetate were all obtained from Sigma. Bromobimanes (monobromobimane, dibromobimane, and monobromotrimethylammoniobimane) were purchased from Molecular Probes (Eugene, OR), and 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid disodium salt were purchased from Fluka (Buchs, Switzerland). Diamide (azodicarboxylic acid bis-dimethylamide), azodicarbonamide, As$_2$O$_3$, CdCl$_2$, and Sb$_2$O$_3$ were obtained from Aldrich (Milwaukee, WI). Stock solutions of DMPS were prepared in sterile water. PAO solutions were prepared in DMSO (Sigma) and gently heated until PAO went into solution. PAO-induced shedding was accomplished by incubating cells with PAO (at 100 nM unless otherwise specified) for 10 min at 37°C. DMP and DMPS were used at a final concentration of 50 $\mu$M unless otherwise stated. Neutrophils were incubated with these reagents for 10 min at 37°C. TAPI-2 (provided by Dr. Roy A. Black, Immunex, Seattle, WA) was prepared in DMSO and used at a final concentration of 100 $\mu$M; it was administered to the cells for 10 min at 4°C before stimulation with fMLF or addition of PAO.

### PAO reversal assays

PAO reversal assays were performed by first preincubating cells with 100 nM PAO for 10 min at 4°C, then either DMP or DMPS was added. Cells were incubated for another 10 min at 4°C, followed by an additional 10-min incubation at 37°C.

### Analysis of surface Ag expression

Direct immunofluorescence labeling of control and treated cells was performed in a final volume of 200 $\mu$l at 10$^6$ cells/ml by incubating cells with mAb for 1 h at 4°C. Leu 8-FITC (IgG2a; Becton Dickinson Monoclonal Antibodies, Lincoln Park, NJ), a fluorescent mAb that recognizes L-selectin, was used at a final concentration of 0.625 $\mu$g/ml. Likewise, Leu 15-PE (IgG2a; Becton Dickinson Monoclonal Antibodies), a fluorescent mAb that recognizes the $\alpha$-subunit (CD11b) of Mac-1, was used at 1.25 $\mu$g/ml. The relative expression of the receptors was quantitated using a FACScan Flow Cytometer (Becton Dickinson).

### Immunophenotyping assay

Control cells and PAO-treated cells (100 nM for 10 min at 37°C) were labeled for surface expression of several epitopes. Direct immunofluorescence labeling of cells was performed for detection of L-selectin and $\beta_2$ integrin with Abs Leu 8-FITC and Leu 15-PE. Indirect immunofluorescence was used to detect the remaining epitopes, including PDI. Cells (1 $\times$ 10$^6$) in 200 ml of HHB were incubated for 40 min at 4°C with appropriate Abs. The Abs were against CD14, CD16 (both at 10 $\mu$g/ml; Dako, Carpinteria, CA), CD43 (8 $\mu$g/ml; IgG2a; Camfolio (Becton Dickinson), San Jose, CA), CD54 (8 $\mu$g/ml; BioSource, Camarillo, CA), PSGL-1 (PL1; IgG1; 10 $\mu$g/ml; a gift from Dr. Rodger McEver, University of Oklahoma, Oklahoma City, OK). After incubation the cells were washed by centrifugation for 10 min at 400 $\times$ $g$ at 4°C. The second Ab, goat anti-mouse IgG-FITC polyclonal Ab (Becton Dickinson Antibodies) at a concentration of 6.25 $\mu$g/ml, was added, and cells were incubated for an additional 20 min at 4°C. After a final wash, the specific labeling for each Ab was analyzed by flow cytometry. Expression of PDI on the cell surface was determined similarly. Anti-PDI mAbs (clone RL90 (IgG2a) and clone RL77 (IgG2b), both 1.5 mg/ml) were obtained from Affinity BioReagents (Golden, CO). Both were used at the final dilution of 5 $\mu$l/100 $\mu$l (10$^5$) cells. Matched isotype control Abs (Coulter, Hialeah, FL) were used to measure any nonspecific staining. The results are reported as the relative mean channel fluorescence.

### PAO time-course experiments

For these experiments, isolated neutrophils, eosinophils, or lymphocytes were warmed to 37°C, and a zero point sample was withdrawn and placed on ice. PAO was then added (1 $\mu$M for neutrophils and eosinophils, and 5 $\mu$M for lymphocytes). Cell samples were withdrawn at 1-min intervals and placed on ice. Thereafter, the cells were labeled for 40 min with Leu 8-FITC for the lymphocyte preparation or with Leu 8-FITC plus anti-VLA-4-PE for the granulocyte population. This permits the simultaneous identification and quantitation of L-selectin on neutrophils and eosinophils as described above.

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## Soluble L-selectin ELISA

Fifty-microliter aliquots of neutrophils suspended in HEPES buffer at $10^6$ cells/ml were treated with 100 nM PAO, 100 nM PAO followed by 50 µM DMPS, 100 µM TAPI-2 followed by 100 nM PAO, 100 nM fMLF, or 100 µM TAPI-2 followed by 100 nM fMLF, with necessary incubations as outlined above. Also included was an untreated control kept at 4°C and a DMSO-treated sample subjected to a 10-min 37°C incubation. Aliquots were then centrifuged, and the supernatants were removed and prepared according to the test protocol of the Bender MedSystems (Boehringer Ingelheim Group, Vienna, Austria) sL-selectin ELISA kit. Neutrophils were resuspended in buffer and labeled for L-selectin expression with Leu 8-FITC (as above), then analyzed by FACScan.

## Neutrophil aggregation measurements

Methods for aggregation measurements have been described previously (6). Briefly, control and treated cells, in a volume of 500 µl at $4 \times 10^6$ cells/ml, were labeled with the nucleic acid stain LDS-751 (Exciton, Dayton, OH) at 0.04 µg/ml for 7 min at 37°C. Samples were equilibrated for 2 min at 37°C under conditions of shear mixing using a small bar magnet ($7 \times 2$ mm; VWR Scientific, Media, PA) above a heated stir device at 500 rpm. Samples were then activated with 0.1 µM fMLF, and data were acquired at specific intervals after stimulation. We report the percentage of cells that formed aggregates.

## Dual population aggregation of neutrophils with ICAM-1 transfectants

This method has been previously described (6). Briefly, a transfected murine melanoma cell line expressing ICAM-1, Uill/E3, was aggregated with neutrophils to test neutrophil integrin activity. For dual population aggregation experiments, Uil1/E3 cells were labeled using a membrane-linked stain, PKH2-GL (Sigma). Labeled Uil1/E3 were suspended in HHB buffer containing 1.5 mM $CaCl_2$ and 0.1% human serum albumin, and 250 µl of labeled Uill/E3 cells at $3 \times 10^6$ cells/ml were combined with 250 µl of LDS-75 (40 ng/ml; Exciton)-labeled neutrophils at $3 \times 10^6$ cells/ml. The singlet and aggregate events were quantitated using FACScan research software. An analysis gate was placed around each specific cluster of events. We report here the percentage of neutrophils that were involved in two-color heterotypic aggregates.

## Induction of L-selectin shedding by anti-PDI Abs

Neutrophils were preincubated in the absence or the presence of anti-PDI mAbs (clone RL90 (IgG2a) and clone RL77 (IgG2b), Affinity BioReagents) or matched isotype control mAbs (Coulter). Five microliters of Ab was added to $4 \times 10^5$ cells in a final volume of 200 µl, then the sample was incubated for 30 min on ice. Following a 10-min incubation at 37°C, the cells were washed with ice-cold HEPES buffer and assayed for L-selectin expression with Leu 8-FITC as described above.

## Induction of L-selectin shedding with bacitracin

Bacitracin (100 mM; Sigma) or purified bacitracin A (a gift from Leo Kesner, Biology Department, State University of New York Health Sciences Center, Brooklyn, NY) stock was prepared in HHB and used to treat neutrophils at a final concentration of 3 mM. To rule out LPS contamination, some neutrophil samples were pretreated for 30 min at 4°C with 20 µg/ml of MY4 (Coulter), a mAb that blocks the LPS receptor CD14 (8). After the 30-min incubation at 37°C, the cells were placed on ice and assayed for L-selectin expression with Leu 8-FITC.

## Interaction of PDI with PAO affinity resin

ThioBond (Invitrogen, San Diego, CA), an agarose-based support covalently modified with PAO, was washed twice with PBS (pH 7.2; Life Technologies, Grand Island, NY). A 500-µl aliquot placed in a 1.5-ml microfuge tube was activated with 1 ml of 20 mM 2-ME (Sigma). The tube was rocked at room temperature for 60 min. The resin was allowed to settle by gravity, and the supernatant was decanted. The resin was washed three times with PBS. PDI (20 µg; Calbiochem, San Diego, CA) was solubilized in 500 µl of PBS and added to the activated ThioBond. The sample was rocked for 90 min at room temperature. The resin was gravity settled, and the PDI solution was decanted and saved. Five subsequent washes were performed. To elute the bound protein, 500 µl of 0.5 M β-ME was added, and the sample was rocked at room temperature for 30 min. The eluate was

Table I. *Thiol oxidizing and blocking reagents induce L-selectin shedding from neutrophils[a]*

| Blocking Reagents | Concentration |
|---|---|
| PAO | <1 µm |
| Aminophenylmercuric acetate | 10 µM |
| Nitroblue tetrazolium | 100 µM |
| Monobromobimane | 100 µM |
| Dibromobimane | 100 µM |
| Hydrogen peroxide | 150 µM |
| DIDS* | 200 µM |
| N-ethylmaleimide | 250 µM |
| Quaternary bromobimane* | 300 µM |
| Iodoacetate | 300 µM |
| Mersalyl acid* | 500 µM |
| Thimerosal* | 500 µM |
| DTNB* | 1 mM |
| PMSF | 1 mM |
| Diamide | 1 mM |
| Azodicarbonamide | 1 mM |
| Iodoacetamide | 10 mM |

[a] The approximate concentrations of the reagent, resulting in complete shedding (as compared to the fMLF-positive control sample) within 10 min is indicated. The reported membrane-impermable reagents are marked with an *.

then collected. Twenty-microliter fractions from the flow-through volume, each wash, elution, and a PDI control were assayed by SDS-PAGE. A control was generated by incubating 500 µl of ThioBond in 1 ml of a 10 mM DTT (Sigma) solution. DTT irreversibly inactivates the resin. PDI (20 µg) was then added to the resin, followed by the washing and elution procedure described above.

## Results

### Sulfhyrdryl reagents regulate L-selectin shedding

We have previously reported on the activation of integrin function by sulfhydryl reactive agents (47, 48). During the course of these investigations we observed that thiol-reactive agents also regulated L-selectin shedding. In general, the oxidizing and thiol-blocking reagents promote shedding (Table I). These include membrane-permeable thiol-reactive iodoacetate, monobromobimane, dibromobimane, 4-aminophenylmercuric acetate, and N-ethylmaleimide. The membrane-impermeable reagents include DTNB, mersalyl acid, thimerosal (sodium ethylmercurithiosalacylate), 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid, and quaternary BBr. Their monothiol reactivity, rather than membrane impermeability, is likely to account for their uniformly low effectiveness. Nitro blue tetrazolium is a superoxide scavenger and, like hydrogen peroxide, a potent oxidant. Azodicarbonamide is a structural analogue of diamide that is well known for its ability to cross-link thiols (49), while PMSF is reactive with thiol nucleophils. In addition, diagnostic inhibitors of enzymes with active site dithiol groups, such as arsenite and $Cd^{2+}$ (50, 51), also induce L-selectin shedding (not shown). Trivalent arsenite ($As_2O_3$) and antimony ($Sb_2O_3$) are the most potent (inducing full shedding in 10 min at about 50 µM), while the divalent cadmium ($CdCl_2$) requires higher concentrations (1 mM). In contrast, high concentrations (5 mM) of the dithiol reducing agents were shown not to induce L-selectin shedding, but, rather, to block shedding when neutrophils were activated with formyl peptide (Fig. 1). Monothiol reducing reagents do not affect the rate of fMLF-induced shedding. We have shown previously that activation of cell adhesion occurs normally (47).

The most potent shedding reagent, PAO (reactive with vicinal dithiols), was examined more closely. Neutrophils shed their L-selectin in response to PAO in a dose-dependent fashion (Fig. 2a). A 10-min incubation with 100 nM PAO reduces L-selectin levels to that comparable with a 5-min treatment with 100 nM fMLF.
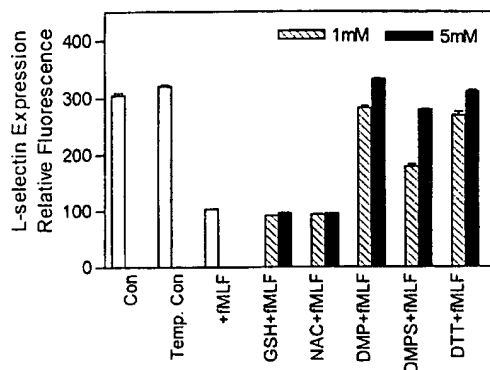
FIGURE 1. Inhibition of fMLF-induced shedding of L-selectin by dithiol reducing agents. Neutrophils were pretreated with the reducing reagents for 5 min on ice before the addition of fMLF (0.1 $\mu$M) and immediately thereafter were incubated for 5 min at 37°C. The reaction was terminated by placing the samples on ice, and the levels of the remaining cell surface L-selectin were determined by labeling with anti-L-selectin-FITC Ab and FACScan analysis. The monothiol reducing agents, glutathione and $N$-acetyl-L-cysteine, did not inhibit L-selectin shedding. High concentrations of dithiol reducing agents were able to inhibit shedding of L-selectin induced by fMLF. Experiments were performed at least three times and were conducted in triplicate. L-selectin levels are reported here as the mean ± SEM of a representative experiment.

Activation-dependent down-regulation of L-selectin is tightly co-ordinated with an increase in the surface expression of the Mac-1 (CD11b/CD18) $\beta_2$ integrin (6). Marginal up-regulation of Mac-1 in response to PAO (Fig. 2b), much less than that induced by activation with fMLF, indicates that PAO induces L-selectin shedding without activating the neutrophils. Similarly, PSGL-1 (a ligand for P- and L-selectin), CD14 (LPS receptor), CD16 (Fc$\gamma$ receptor), CD43 (sialophorin; a major sialoglycoprotein shown to interact with ICAM-1), and CD54 (ICAM-1) were not affected by 100 nM PAO (Fig. 2b).

Neutrophils also shed their L-selectin when whole blood was treated with PAO (Fig. 2c), with 50% shedding at 20 min with 1 $\mu$M PAO. The requirement for an increased concentration of PAO in whole blood compared with the purified neutrophil populations probably reflects an increased number of PAO-reactive sites present in whole blood.

*Reversibility of PAO binding*

The dithiol, heavy metal chelating compound DMP was developed as an antidote for the arsenical war gas and has been extensively used for treatment of arsenical or mercury poisoning (52, 53). DMP (Fig. 3) is able to reverse the binding of PAO to its target (36). It is thought that DMP competes for PAO on the PAO-protein complex by reducing the vicinal sulfhydryls, stripping PAO from its target protein(s), and forming a stable, soluble chelate (52) (Fig. 3).

To determine whether PAO binding was DMP reversible, neutrophils were treated with 100 nM PAO for 10 min at 4°C. Unbound PAO was removed by centrifuging the cells, aspirating the supernatant, and resuspending the cells in fresh buffer containing 50 $\mu$M DMP. The suspension was left at 4°C for an additional 10 min, incubated at 37°C for 10 min, then labeled with mAb for L-selectin. Under these conditions L-selectin expression remained near control levels (Fig. 4a). DMP at this concentration was not able to inhibit fMLF-induced shedding of L-selectin, indicating a selective reversal of PAO effects by DMP rather than an inhibition of fMLF signaling or the proteolytic cleavage of L-selectin.
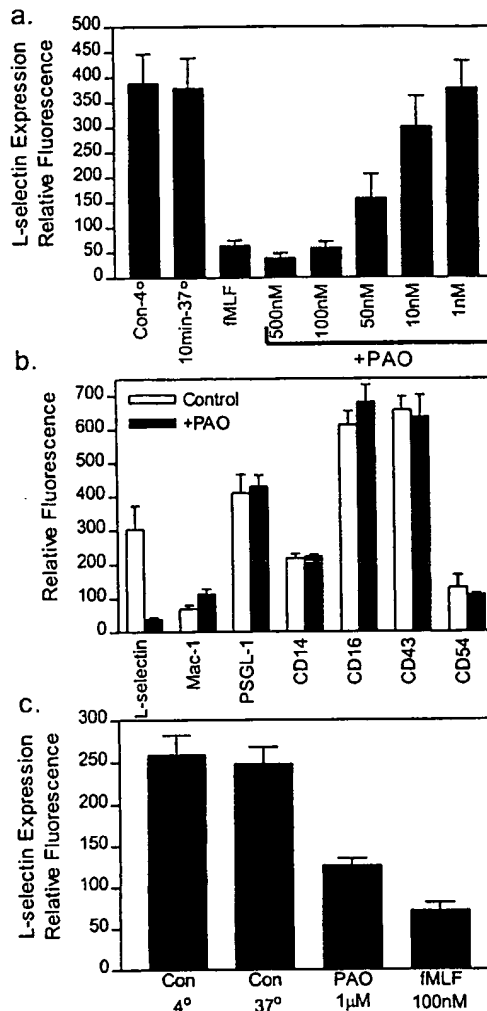


FIGURE 2. Effect of PAO on neutrophil adhesion molecules. a, PAO induces L-selectin shedding from neutrophils in a dose-dependent manner. Control-4°C, a neutrophil population that has remained at 4°C since isolation; Control-10 min @37°C, an untreated population that underwent a mock 10-min incubation along with the PAO- and fMLF-treated cells. b, Phenotypic analysis of PAO-treated neutrophils. PAO (100 nM) does not appreciably up-regulate Mac-1 or induce the shedding of other cell surface molecules known to undergo proteolytic cleavage. c, Activity in whole blood. Peripheral venous blood was diluted 1/10 with HHB buffer and treated with 1 $\mu$M PAO for 20 min at 37°C. L-selectin expression was measured by subsequent FACScan analysis of the LDS-751 and Leu 8-FITC labeled cells. The mean channel number of fluorescence is reported ±SEM. Experiments were performed two or three times with duplicate or triplicate samples in each.

DMPS (Fig. 3) is a membrane-impermeable analogue of DMP that has previously been used to define a site of PAO action with respect to the surface of the plasma membrane (54). Lower concentrations (50 $\mu$M) of DMPS, like the membrane-permeable DMP, did not interfere with the fMLF-induced shedding. DMPS was, however, still able to inhibit the PAO-induced shedding of L-selectin from neutrophils (Fig. 4a). This suggests that the mechanism by which PAO causes L-selectin to be shed is an extracellular process and is not due to intracellular signaling. Several other thiol reagents noted in Table I, reported to be membrane impermeable, induced L-selectin shedding. In contrast, most other previously reported PAO-induced events in neutrophils appear to be related to intracellular actions (39, 55, 56). Neither DMP nor
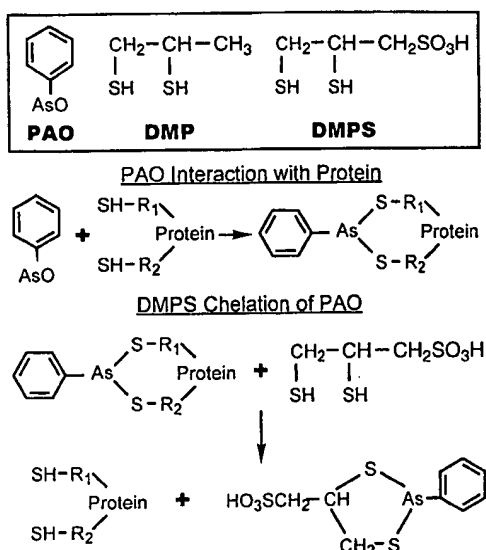
**FIGURE 3.** DMP, and DMPS structures. PAO interacts covalently with vicinal dithiol-containing proteins. The addition of DMP or DMPS effectively chelates PAO and restores the protein to its original reduced dithiol state.
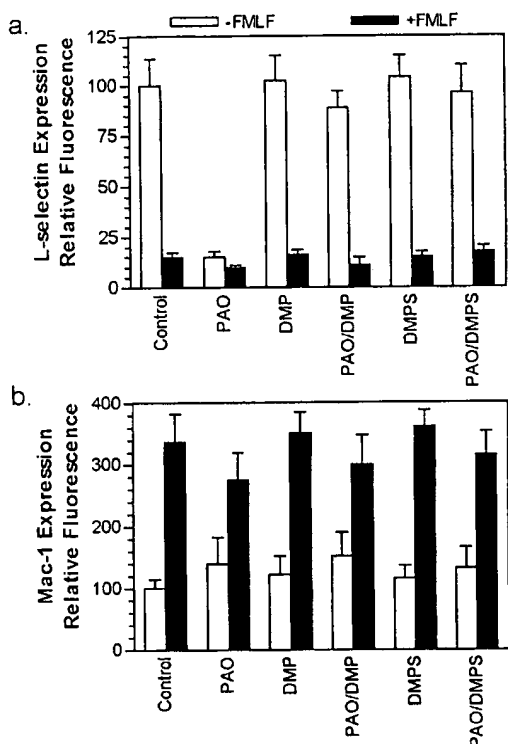


**FIGURE 5.** Time course of PAO-induced shedding of L-selectin from neutrophils, eosinophils, and lymphocytes. Isolated leukocyte populations were warmed to 37°C, and PAO was added at 1 $\mu$M for the neutrophil and eosinophil populations and 5 $\mu$M for the lymphocytes. Duplicate 100-$\mu$l samples were removed at 1-min intervals and placed on ice. Controls (filled symbols) at 10 min represent untreated populations that underwent the incubation at 37°C along with the PAO-treated cells. Anti-L-selectin Ab Leu 8-FITC was subsequently added to the cells on ice, and cell-associated L-selectin levels were measured using the FACScan. Anti-CD49-PE, also added to the granulocyte preparation, was used to simultaneously identify eosinophils in the granulocyte population. Mean channel number is reported. The experiments were performed three or more times, each in duplicate.

DMPS had adverse effects on Mac-1 (Fig. 4b). These compounds did not up-regulate the integrin on their own and did not interfere with fMLF-induced up-regulation. As a result, we conclude that it is unlikely that either DMP or DMPS interacts with the pathway that regulates L-selectin shedding. We envision that at low concentrations DMP and DMPS prevent the PAO-induced shedding of L-selectin by abstracting PAO from its distinct binding site(s) on the cell surface.

*Rate of L-selectin cleavage in neutrophils, eosinophils, and lymphocytes*

To determine whether PAO had a similar effect on L-selectin levels in other leukocytes, we examined L-selectin expression over time in lymphocytes and eosinophils along with neutrophils (Fig. 5). Eosinophils initially expressed lower levels of L-selectin and shed this molecule in response to PAO more slowly than neutrophils within the same granulocyte population. Similarly, lymphocytes also showed a lower basal level of L-selectin expression and, even at increased concentrations of PAO, a considerably slower rate of PAO-induced L-selectin shedding compared with neutrophils. PAO (5 $\mu$M), however, induced complete L-selectin release from lymphocytes in 30 min. When the incubation was conducted in the absence of PAO, none of these three leukocyte types experienced significant spontaneous L-selectin shedding (Fig. 5). These results show that although expressing different initial levels of L-selectin and responding with different rates of shedding, PAO induces shedding in neutrophils, eosinophils, and lymphocytes.

*Inhibition of neutrophil aggregation by PAO*

To verify the functional integrity of neutrophils treated with PAO, we examined the ability of neutrophils to aggregate with one another. We have previously shown that homotypic aggregation,



**FIGURE 4.** Inhibition of PAO-induced shedding of L-selectin. a, DMP and DMPS (at a final concentration of 50 $\mu$M) do not modulate L-selectin expression on their own; however, both reagents interfere with PAO (100 nM) induced shedding of L-selectin without inhibiting fMLF (100 nM) induced shedding of L-selectin. Data are normalized, with the control (without fMLF) representing 100%. Error bars indicate the SEM. b, Neither PAO, DMP, nor DMPS up-regulated Mac-1 or inhibited fMLF induced up-regulation. The experiment was repeated three times with duplicate determinations for each.
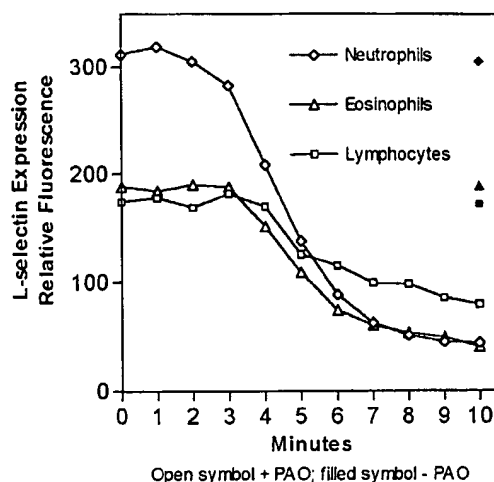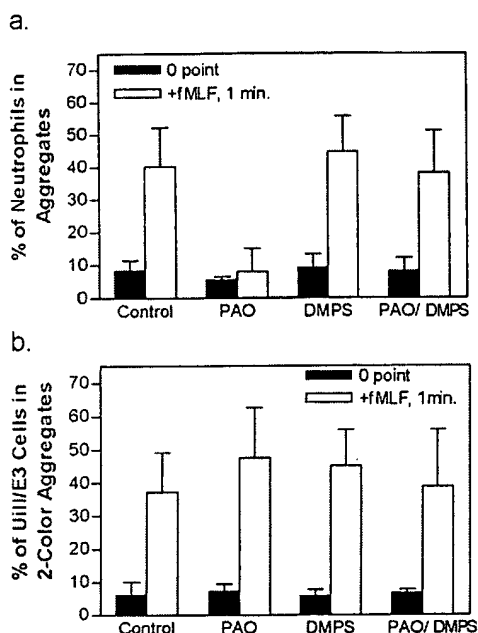
a.



b.



FIGURE 6. Measurement of neutrophil homotypic and heterotypic aggregation. a, Neutrophil homotypic aggregation was inhibited in 100 nM PAO-treated cells. DMPS (30 $\mu$M) alone had no affect on aggregation and was able to interfere with PAO-induced inhibition of aggregation presumably by removing bound PAO from the cell surface and preventing PAO-induced shedding of L-selectin. Aggregates were measured before stimulation (0 point) and at 1 min after stimulation with 100 nM fMLF. The number of neutrophils in aggregates and SEM is reported. b, ICAM-1-transfected cells were used to test the effect of PAO on integrin function. Data are expressed as the percentage of Uil1/E3 cells that aggregated with activated neutrophils at 1 min after stimulation. Isolated neutrophils and Uil1/E3 mouse melanoma cells transfected with human ICAM-1 were prepared and labeled as described in *Materials and Methods*. Neutrophils were pretreated with PAO, DMPS, or both. The cells were washed once and combined at 3 X 10$^6$cells/ml each, then stimulated with fMLF. The data represent two experiments, with duplicate determinations in each.

which occurs when neutrophils are exposed to fMLF or leukotriene B4 under shear stress (6, 7), involves two sequential steps that are analogous to leukocyte-endothelial cell adhesion. The first step is a low affinity interaction between neutrophil L-selectin and its mucin counterstructure PSGL-1 on the opposing neutrophil (7). The second step is a high affinity adhesion between a $\beta_2$ integrin (CD18) and its neutrophil ligand, most likely ICAM-3 (18, 57). Accordingly, the aggregation of PAO-treated neutrophils was inhibited (Fig. 6a) due to the loss of L-selectin. Although DMPS alone at low micromolar concentrations did not have an adverse effect on aggregation, DMPS was able to rescue the ability of the cells to aggregate, presumably by removing the PAO from the critical target protein. This allowed L-selectin to remain on the surface and to initiate the aggregation process upon stimulation with fMLF.

*Effects of PAO on $\beta_2$ integrin function*

To verify that the absence of aggregation in PAO-treated neutrophils was due to the loss of L-selectin and was not the result of PAO interfering with intracellular signaling or integrin activation, a murine melanocyte cell line transfected with ICAM-1 was used to assess $\beta_2$ integrin function (Fig. 6b). The adherence of neutrophils with the ICAM-1-transfected cells is dependent solely on the integrin step (58). Although treatment with 100 nM PAO inhibited
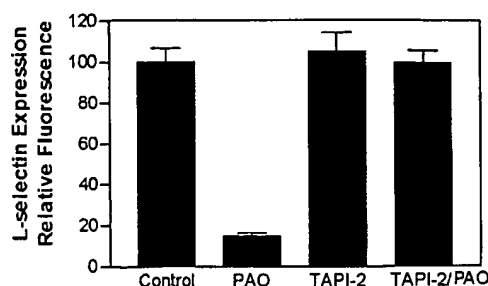


FIGURE 7. TAPI-2 inhibits PAO-induced shedding of L-selectin. Cells were pretreated with 100 $\mu$M TAPI-2 for 10 min at 4°C. PAO (100 nM) was then added, and cells were incubated for 10 min at 37°C. L-selectin expression was normalized to control levels, set to represent 100%. The experiment was performed twice, with triplicate samples in each. The mean and SEM are given.

homotypic neutrophil aggregation (Fig. 6a), it did not inhibit the adhesion of neutrophils to target cells (Fig. 6b). This demonstrated that at this concentration PAO treatment does not interfere with the signaling pathways that lead to an increase in the adhesive competence of neutrophil integrins.

*Inhibition of PAO-induced shedding of L-selectin by TAPI-2*

TAPI-2, a hydroxamate-based inhibitor of matrix metalloproteases, has previously been shown to inhibit the activation-induced shedding of L-selectin from neutrophils, eosinophils, and lymphocytes (18, 29–31). Here we show that TAPI-2 also inhibits PAO-induced shedding in neutrophils (Fig. 7). Thus, TAPI-2 appears to be able to inhibit the activation-independent release of L-selectin as well as the activation-dependent release.

*ELISA for soluble L-selectin*

To confirm that the L-selectin analysis by flow cytometry represented shedding from the neutrophil surface, an sL-selectin ELISA was performed on cell supernatants. Neutrophils were treated with 100 nM PAO, 100 nM PAO followed by 50 $\mu$M DMPS, 100 $\mu$M TAPI-2 followed by 100 nM PAO, 100 nM fMLF, or 100 $\mu$M TAPI-2 followed by 100 nM fMLF. Control samples and samples containing TAPI-2 displayed negligible levels of sL-selectin ($\ll$0.20 ng/ml). The PAO/DMPS samples registered slightly higher reading at ~0.4 ng/ml, while the PAO and fMLF samples displayed sL-selectin levels in the 1.6–2.0 ng/ml range. This indicates that PAO, like fMLF treatment, results in the release of the L-selectin molecule into the medium. As further conformation, the cells from which the supernatants were taken were subsequently labeled for L-selectin surface expression. The results mimicked those shown in Figs. 2a, 4a, and 7, in which control, DMPS, and TAPI-2 samples maintained near normal levels of cell surface L-selectin expression, while PAO- and fMLF-treated cells displayed minimal levels of L-selectin.

A hypothesis was developed in which extracellular PAO regulates the susceptibility of the L-selectin molecule to a constitutively active, TAPI-2-inhibitable, protease. We postulated that PDI (43–45), known for its ability to rearrange disulfide bonds within a variety of substrate proteins, could promote an interchange between its thiols and the disulfide bonds of the 24 cysteine residues of L-selectin. To explore this, we first determined that PDI is indeed expressed on the neutrophil cell surface (Fig. 8a). Additionally, two anti-PDI monoclonals, both previously reported to inhibit PDI activity (59, 60), were found to induce L-selectin shedding
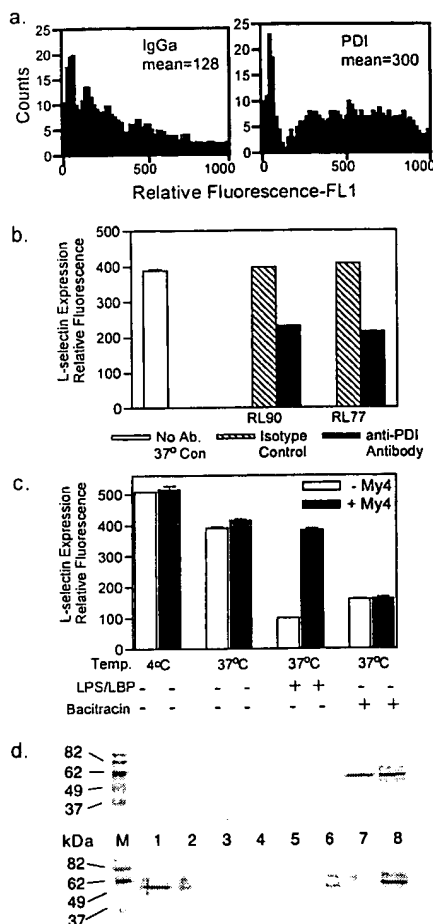
4126 PHENYLARSINE OXIDE INDUCES L-SELECTIN SHEDDING

PHENYLARSINE OXIDE INDUCES L-SELECTIN SHEDDING

a.

IgGa
mean=128

PDI
mean=300

Counts

25 20 15 10 5 0
0   500   1000   0   500   1000

Relative Fluorescence-FL1

b.

L-selectin Expression
Relative Fluorescence

500 400 300 200 100 0

RL90    RL77

No Ab. 37°Con     Isotype Control     anti-PDI Antibody

c.

L-selectin Expression
Relative Fluorescence

500 400 300 200 100 0

- My4
+ My4

Temp.    4°C    37°C    37°C    37°C
LPS/LBP   -   -    -  -    + +    - -
Bacitracin  -   -    -  -    - -    + +

d.

82
62
49
37

kDa  M  1  2  3  4  5  6  7  8

82
62
49
37

FIGURE 8. A role for PDI in L-selectin shedding. a, Neutrophils express cell surface PDI. Neutrophils were labeled with the monoclonal anti-PDI Ab (clone RL90 or the isotype control (IgG2a)) and washed, and the bound anti-PDI was detected by the secondary, goat anti-mouse FITC Ab. A representative FACScan histogram is shown, demonstrating a fluorescence increase in the anti-PDI-labeled cells of about 170 channels over that of the IgGa isotype control. The experiment with this clone and with another anti-PDI clone, RL77 (an IgG2b isotype), was repeated in duplicate at least five times, and comparable results were obtained. b, Inhibition of PDI activity with anti-PDI mAbs induces L-selectin shedding. Neutrophils were incubated in the presence of anti-PDI Abs RL90, RL77 (filled bars), or their isotype control Abs, IgG2a or IgG2b, respectively (hatched bars), for 30 min on ice (2 $\mu$l/10$^5$ cells) to allow Abs to bind, and then were incubated at 37°C for 15 min. Cells were assayed for L-selectin expression with Leu 8-FITC, and means are shown. Analogous experiments, performed four or more times, were conducted in duplicate. c, Bacitracin induces L-selectin shedding in an LPS/LPS binding protein-independent manner. Cells were preincubated for 30 min on ice with or without the LPS receptor CD14 blocking Ab My4 (20 $\mu$g/ml). Bacitracin (3 mM), the standard concentration used to inhibit cell surface PDI activity, or LPS/LPS binding protein (10 ng/ml) were added where indicated and incubated, along with duplicate control samples, at 37°C for 30 min. Cells were assayed for L-selectin expression with Leu 8-FITC, and the means are shown. Experiments were conducted three times in duplicate. d, PDI specifically binds to PAO affinity resin (ThioBond). SDS-PAGE analysis of flow-through (lane 1), washes (lanes 2–6), elution (lane 7), and PDI control (lane 8). The upper gel represents fractions collected from ThioBond resin activated with 20 mM $\beta$-ME, then incubated with 20 $\mu$g of PDI. The majority of PDI was bound to the resin, only coming off when eluted with 0.5 M $\beta$-ME (lane 7). The lower gel represents fractions from ThioBond inactivated with 10 mM DTT. Resin treated this way was not able to bind PDI. The initial flow-through contained most of the PDI (lane 1).

(Fig. 8b). We further verified the involvement of PDI using another known inhibitor of PDI activity, bacitracin (Fig. 8c). This antibiotic inhibits PDI, but not thioredoxin, the other enzyme also present at the cell surface and known to catalyze oxido-reduction reactions (60). LPS receptor CD14-blocking Abs (My4) were used to verify that LPS contamination of bacitracin was not involved in the induction of L-selectin shedding from these bacitracin-treated neutrophils. Moreover, bacitracin-induced shedding was not due to neutrophil activation, which would result in the characteristic quantitative up-regulation of the cell surface $\beta_2$ integrins (data not shown). These results were confirmed using bacitracin further purified by Dr. Kesner (State University of New York). Lymphocytes also express PDI on the cell surface (61) (as confirmed by us) and respond to anti-PDI Abs by shedding L-selectin, albeit much more slowly than neutrophils. Lymphocytes only respond to prolonged (>30-min) bacitracin treatment (data not shown). Finally, we have obtained the first direct evidence for the interaction of PAO and PDI. ThioBond resin (Invitrogen), an agarose-based support covalently attached to PAO, specifically binds purified PDI (Fig. 8d). ThioBond did not bind BSA, and resin inactivated by treatment with DTT was not able to bind PDI.

## Discussion

### L-selectin shedding and the sheddase

The predominant mechanism for regulating L-selectin-mediated adhesion is its proteolytic shedding from cell surfaces during an immunological or inflammatory response (15). Several laboratories have concluded that the L-selectin sheddase is a constitutively active protease, most likely identical with TNF-$\alpha$ converting enzyme (28), and that it is the conformational status of L-selectin molecule that determines the susceptibility of the L-selectin molecule to the proteolytic cleavage (26, 27, 32). It has been postulated that ligand binding or cellular activation induces the protease-susceptible conformation in the membrane-proximal region of L-selectin. Cellular activation has also been reported to induce a transient conformational change in L-selectin molecules that correlates with an increase in L-selectin avidity for its ligand PPME (polyphosphomannan monoester core) (62). Thus, ligand and cell activation-induced changes in L-selectin conformation may be a mechanism that ensures that the rapid increase in L-selectin receptor avidity is efficiently down-modulated through its subsequent proteolytic release (62).

### PAO and sulfhydryl regulation

We have followed a lead that suggests regulation of shedding through extracellular sulfhydryl chemistry (Table I and Fig. 1). Moreover, purified neutrophils and eosinophils respond rapidly to low micromolar doses of PAO without compromising the signaling and adhesive functions of other adhesion molecules such as Mac-1 (Figs. 2, 4, and 6). Lymphocytes also respond to PAO by shedding their L-selectin, although they require somewhat higher concentrations and longer incubation times (Fig. 5). Homotypic aggregation, here used as a model for the L-selectin-dependent adhesive processes and known to play a physiologic role in inflammatory amplification, is inhibited by PAO (Fig. 6). Other L-selectin-dependent interactions, such as the recruitment of leukocytes to the inflammatory sites and lymphocyte recirculation through the lymph nodes, are expected to be profoundly affected by the PAO-induced L-selectin loss.

Because the membrane-impermeable PAO-reversing reagent DMPS blocks PAO-induced L-selectin release, the critical PAO

target protein is likely to reside on the outside of the plasma membrane (Fig. 4). This extracellular location of the L-selectin shedding regulatory protein is substantiated by the analogous, although far less potent, effect of the membrane-impermeable monothiol-reactive reagents. The higher specificity of the dithiol-reactive PAO combined with its extracellular site of action provide an opportunity to cause L-selectin shedding with membrane-impermeable analogues of PAO. Restricting PAO access to the cell surface promises to minimize toxicities associated with intracellular PAO.

*Mechanism of PAO action*

Although we have not yet formally excluded the idea that PAO interacts directly with L-selectin, we have obtained evidence for a regulatory molecule such as PDI (Fig. 8). Inhibition of PDI activity by DTNB, anti-PDI Abs, and bacitracin lead to L-selectin shedding. If cell surface PDI could act as a regulatory protein that retains L-selectin in a noncleavable conformation, then the inhibition of its oxido-reductive capacity by PAO, through interaction with a thioredoxin-like active site Cys-Gly-His-Cys, is expected. Similarly, mono-thiol-reactive N-ethylmaleimide and DTNB, both inducers of L-selectin shedding, are routinely used to block PDI activity, albeit at high concentrations (63). Although PAO has not yet been reported to block PDI active sites, it is known to cross-link the homologous active site in thioredoxin (64). PDI is a sub-unit of the tri-iodothyronine receptor (65), and the recombinant rat tri-iodothyronine receptor has been shown to bind specifically to a PAO affinity column (66). Additionally, we have shown that purified PDI specifically binds to a PAO affinity column, providing further evidence for an interaction between PDI and PAO.

One remaining speculation is that L-selectin is a substrate for PDI. PDI is known to catalyze disulfide bond interchange in a spectrum of substrate proteins, and this isomerase function depends on the integrity of the vicinal-dithiol active sites. PDI is also a chaperone whose function does not depend on its isomerase activity (43–45). The chaperone activity is thought to be due to PDI binding to proteins that have a tendency to aggregate in the denatured state by promoting the correct folding of the protein. PDI is a critical component of protein complexes such as the α-subunit of prolyl-hydroxylase, N-glycosyl transferase, and the triglyceride transfer protein complex, where it is required to maintain triglyceride transfer protein in catalytically active form and to prevent its aggregation (67).

Despite its Lys-Asp-Glu-Leu endoplasmic reticulum retention signal, PDI has been detected on the surface of many cell types, including hepatocytes, platelets, and lymphocytes (60, 61, 68, 69), and is implicated in many cell surface processes. A PDI homologue, cognin, plays a role the adhesion-dependent aggregation of retinal cells (70). PDI modulates the conformation of thrombospondin where the isomerization of disulfide bonds is likely to have a profound effect on its ligand binding and adhesive capacity (71). Couet et al. have demonstrated that cell surface PDI is involved in the shedding of human thyrotropin receptor ectodomain (60). By analogy, our results implicate PDI in the release of L-selectin. One crucial difference is that it is the inhibition of neutrophil PDI that permits L-selectin shedding. Moreover, L-selectin shedding can be induced by treatment with the same PDI-blocking reagents (DTNB, bacitracin, and anti-PDI Abs) that impede the release of the thyrotropin receptor. It remains to be determined whether this PDI-mediated mechanism operates independently or is a part of the calmodulin-controlled, L-selectin shedding pathway (33).

Neutrophils contain appreciable immunoreactive PDI in their specific granules, and degranulation with PMA treatment releases
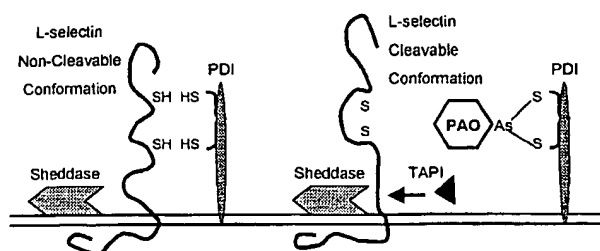


FIGURE 9. Regulation of L-selectin shedding. Reduced cell surface PDI constitutively maintains L-selectin in the reduced, noncleavable conformation. Chemical blockade or direct oxidation of the PDI vicinal dithiol active sites led to the formation of a critical disulfide bridge within the L-selectin molecule. The resulting conformation change in L-selectin permitted cleavage by the sheddase. In the presence of TAPI, L-selectin shedding was blocked.

PDI into the medium (72). We now show that PDI is present on the resting neutrophil cell surface. Neither the oxidative status of the PDI Cyc-x-y-Cys active sites, the impact of cell activation, nor the role of the released PDI in shedding of L-selectin is known.

Because L-selectin initiates the interaction of leukocytes with activated endothelium, L-selectin appears to play a pivotal role in inflammatory disease. These include acute respiratory distress syndrome, ischemia-reperfusion injury that follows myocardial infarction and stroke, the pathogenesis of multiorgan failure that follows sepsis, and the diseases of eosinophilic inflammation, such as asthma and dermatitis. Cell surface PDI-blocking agents, such as impermeable PAO analogues that interfere with both leukocyte adhesion to the endothelium and leukocyte-leukocyte interactions, could play an important role in limiting or perhaps preventing damage in acute as well as chronic inflammatory diseases. Furthermore, HIV-induced $CD4^+$ lymphocyte depletion may be due to L-selectin signaling (14). Abrogation of this signaling by shedding L-selectin might mitigate the decrease in $CD4^+$ cell count that is typical of late stages of AIDS pathogenesis.

In summary, we present our model, illustrated in Fig. 9. This model proposes a novel mechanism by which the cellular chaperone and oxido reductase, PDI, regulates the susceptibility of leukocyte L-selectin to shedding. Cell surface PDI constitutively acts upon L-selectin to maintain disulfide bonds in the reduced, noncleavable state. Blockade of PDI function permits reversion of L-selectin to the oxidized, cleavable conformation. In the presence of the sheddase inhibitor, TAPI-2, L-selectin is retained on the cell surface. We speculate that following physiological activation, L-selectin conformation is modulated by oxidation of its critical sulfhydryls, rendering it sensitive to proteolysis. Our preliminary data suggest that reactive oxygen and/or nitrogen species, released by activated cells, might modulate PDI activity under physiologic conditions. This PDI-mediated mechanism, analogous to that which mediates chloroplast translational activation (73), offers a simple, extracellular switch for the regulation of L-selectin shedding.

## Acknowledgments

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# HIV entry: are all receptors created equal?

CD4 is almost universally required for HIV to enter cells. A mutable disulfide bond of CD4, however, can influence the permissiveness of cells to HIV infection.

MARK A. GOLDSMITH[1] AND ROBERT W. DOMS[2]

Highly active antiretroviral therapy has led to profound and prolonged reductions in HIV viremia in many individuals. However, a multitude of factors—including drug-resistant virus strains, difficult drug regimens and significant side effects—call for the development of new classes of antiretroviral compounds. Extensive basic research efforts in this area have led to the identification of the cell surface receptors required by HIV to infect cells, structure-based insights and an enhanced appreciation of the conformational changes undergone by the viral envelope glycoproteins (Env) as a consequence of receptor engagement. This knowledge base has driven the design of small molecule inhibitors that block one of several steps in the virus-entry pathway. Several entry inhibitors are now under clinical development, and an improved understanding of the virus-entry process should make it possible to predict treatment success, optimize drug combinations and elucidate and manage the mechanisms of resistance to such drugs. In this issue of *Nature Immunology*, Matthias *et al.* introduce an additional wrinkle to the established HIV-1 infection paradigm by suggesting a higher level of complexity[1].

To infect a cell, the trimeric Env protein of HIV-1 first binds to CD4, a step that triggers structural alterations in Env that enable it to engage a coreceptor, usually the chemokine receptors CCR5 or CXCR4[2]. Coreceptor binding also induces definitive conformational changes in Env that allow it to mediate fusion between the viral and host cell membranes. The cellular distribution of the virus receptors *in vivo*, coupled with viral heterogeneity in coreceptor preferences, explains a great deal about HIV tropism: the ability of a virus strain to infect particular cell types. Although coreceptor use varies between virus strains, the use of CD4 is a nearly universal feature of HIV-1 strains. Matthias *et al.* show, however, that there may be variability in CD4 structure within and between

cell types and that this heterogeneity may influence the ability of HIV-1 to gain entry to the host cell cytoplasm[1]. More generally, this study serves as an additional example of the complexities that underlie HIV-1 infection.

CD4 is expressed at relatively high amounts on helper T cells and most thymocytes. It binds to class II major histocompatibility complex (MHC) and enhances antigen-driven T-cell responses. A type I integral membrane protein, CD4 contains four immunoglobulin-like domains (D1–D4), three of which (D1, D2 and D4) contain an intramolecular disulfide
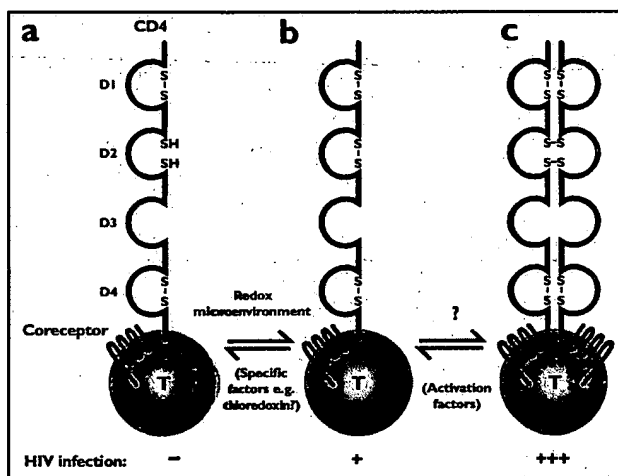


**Figure I. Regulation of HIV infectivity by the redox state of the CD4 D2 domain.** At least three configurations of CD4 have been identified: (a) monomeric with free thiols in D2; (b) monomeric with intramolecular disulfide bond within D2; and (c) dimeric with intermolecular disulfide bond (D2-D2), for which a non-covalent dimer may be a transition state. The relationship among these forms, the key factors regulating interconversion, and the precise impact on modulating susceptibility of HIV are not well understood. One speculative scheme is depicted, which indicates the need to clarify key mechanistic aspects. In particular, the relative contributions of redox-dependent CD4 dimerization versus conformational effects in facilitating HIV entry remain to be established.

bond. HIV-1 Env binds to the D1 domain, which results in a significant conformational change in Env without much effect on the structure of CD4 itself[3]. A somewhat unusual aspect of the CD4 structure is that the disulfide bond in the D2 domain has high strain energy relative to most other disulfide bonds, making it more prone to reduction. Through the use of membrane-impermeable reducing and labeling

reagents, Matthias *et al.* show that a fraction of cell surface CD4 molecules contain one or more free thiols and that this is due to the D2 disulfide bond being redox-active on the cell surface[1]. Although chemical agents that labeled the D2 thiols evidently had no effect on Env binding *per se*, HIV infection was inhibited nonetheless, implying that exchange of the D2 disulfide bond is required for efficient HIV-1 infection[1]. Importantly, infection by a CD4-independent HIV-1 variant was not inhibited by reduction of this bond, providing an important specificity control[1].

The presence of both oxidized and reduced forms of CD4 on the surface of T cells and macrophages shows that this HIV-1 receptor exhibits at least some degree of structural or conformational heterogeneity. Likewise, both CCR5 and CXCR4 can be expressed on the cell surface in conformationally distinct states, as revealed by epitope-mapping studies[4,5]. If HIV makes use of some receptor conformations more efficiently than others, then the mere presence of CD4 and a coreceptor may be insufficient to confer permissiveness for virus infection: other factors may affect the final outcome of the interaction. For example, membrane fusion by an infecting virion is a cooperative process; it involves multiple Env proteins as well as multiple CD4 molecules and chemokine receptors that, in concert, lead to the formation of a fusion pore[2]. Therefore, receptor density is an important factor that influences virus infection[6]. In addition, if receptors preferentially partition into lipid rafts or associate with each other, this could also affect virus infection by leading to local areas on the cell surface that are enriched in virus receptors[7]. Post-translational modification of coreceptors also can affect virus entry, as sulfation of both the CCR5 and CXCR4 NH₂-terminal domains leads to more efficient interactions with Env[8]. Finally, there are differences in how virus strains interact with both

CD4 and coreceptors, and this, too, can influence virus infection. Therefore, even though the presence of CD4 and an appropriate coreceptor are needed for HIV-1 infection, there are a number of local factors that can play a significant role in governing whether infection is successful in a given context, and not all receptor molecules are equally competent to support Env-induced membrane fusion.

The results of Matthias et al. thus suggest that the D2 domain of CD4 exists in oxidized and reduced states[1]. These redox states are probably in an equilibrium influenced by local or cell-autonomous factors; this suggests that HIV-1 entry occurs preferentially via receptors that are passing, at least transiently, through the oxidized state (Fig. 1). The finding that binding of Env to CD4 is not influenced by these states is provocative. A detailed quantification of binding kinetics is required to verify the validity of the interpretation of saturation binding experiments, which implied that the fusion process itself is regulated by obscure post-binding processes involving D1-D2 interactions or more long-range conformational effects. Alternatively, the binding avidity may

be sufficiently enhanced by the dimerization of oxidized CD4 (via intermolecular D2-D2 disulfide bonds) to increase the probability of successful HIV-1 binding, making oxidized CD4 a critical initiator of the fusion cascade. Moreover, for a post-translational modification or conformational heterogeneity to have a significant impact on virus entry, it would likely have to affect a large fraction of cell surface CD4 molecules. Therefore, careful quantification of the reduced and oxidized pools is needed. Interestingly, because multiple CD4-binding events are needed to activate individual Env trimers, a CD4 molecule that binds Env but that does not support subsequent conformational changes could interfere with virus infection in a trans-dominant manner.

It remains to be determined to what extent these results have implications outside the virologist's laboratory. For example, does modulation of the oligomerization or conformation state of CD4 influence the biology of the immune system per se, such as during CD4+ T cell activation? More generally, are other molecules that contain immunoglobulin folds or related secondary structures similarly

redox-active? Finally, is there an anti-HIV therapeutic opportunity within this baroque regulation, as suggested by the Matthias et al.? To date, successful antiretrovirals have targeted essential and dominant steps in the viral life cycle, and this new speculation may represent either an important paradigm shift toward subtle points of intervention or a mere curiosity. As is often the case, bedside evaluation may turn out to offer the only reliable pronouncement regarding this bench discovery.

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# Shc: a dominant player after ten seasons

Ed Palmer

A role for Shc in T cell development was controversial. Two different genetic approaches now show Shc plays a nonredundant and essential function in pre-TCR signaling.

T cell activation and T cell development are dependent on the generation and propagation of signals from cell-surface receptors. Over the past 20 years, a great deal of effort has gone into delineating the signaling pathways in T cells and thymocytes in order to understand how the diverse responses of T cells (for example, activation, anergy and apoptosis) and thymocytes (for example, positive and negative selection) are controlled. Although enormous progress has been made in terms of identifying signaling intermediates expressed in thymocytes and T cells, immunologists still do not completely grasp how the numerous and concurrently activated signaling pathways are integrated to produce a dependable and biologically relevant response. It seems reasonable to ask whether every molecule that is activated after engagement of the T cell receptor (TCR) is actually required or is even relevant for a particular cellular response. For the signaling adaptor Shc,

this question has now been answered by Ravichandran and colleagues in this issue of Nature Immunology[1].

The Shc family of prototypic adaptor molecules is comprised of three homologous genes. Shc1 (which encodes ShcA) was identified 10 years ago by screening a cDNA library at low stringency with a probe encoding an SH2 domain. Shc1 is ubiquitously expressed, whereas Shc2 and Shc3 (which encode ShcB and ShcC, respectively) are limited to the central nervous system. Because only ShcA is expressed in lymphocytes, immunologists have focused on this family member. ShcA consists of three isoforms, p66, p52 and p46, which are splicing variants of a single gene. All the Shc family members have an $NH_2$-terminal phosphotyrosine binding (PTB) domain followed by a collagen homology (CH) domain and then a COOH-terminal SH2 domain (Fig. 1). A database search revealed that 35 proteins contained both PTB

and SH2 domains. Although this number is relatively small, proteins with this modular structure can be found in Drosophila species and Caenorhabditis elegans. Signaling adaptors with this structure are old and have been conserved over a long period of evolution[2].

By virtue of its PTB and SH2 domains, ShcA can bind to activated receptors that have been tyrosine-phosphorylated. Subsequently, tyrosine residues in the CH1 domain of Shc are phosphorylated and Shc is able to recruit Grb2 to the growing signaling complex. Finally, Grb2 binds the Ras activator Sos, which in turn activates Ras and ultimately leads to the activation of the mitogen-activated protein kinase (MAPK) pathways. The attraction of Shc as a signaling adaptor is that it can link activation of a surface receptor to Ras, a key element in the activation of many diverse cell types (Fig. 1). The general importance of Shc is demonstrated by the fact that ShcA-deficient mice die by

# Disulfide exchange in domain 2 of CD4 is required for entry of HIV-1

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**CD4, a member of the immunoglobulin superfamily of receptors that mediates cell-cell interactions in the immune system, is the primary receptor for HIV-1. The extracellular portion of CD4 is a concatenation of four immunoglobulin-like domains, D1 to D4. The D1, D2 and D4 domains each contain a disulfide bond. We show here that the D2 disulfide bond is redox-active. The redox state of the thiols (disulfide *versus* dithiol) appeared to be regulated by thioredoxin, which is secreted by CD4+ T cells. Locking the CD4 and the thioredoxin active-site dithiols in the reduced state with a hydrophilic trivalent arsenical blocked entry of HIV-1 into susceptible cells. These findings indicate that redox changes in CD4 D2 are important for HIV-1 entry and represent a new target for HIV-1 entry inhibitors.**

CD4 is a member of the immunoglobulin (Ig) superfamily that binds to class II major histocompatibility complex (MHC) to enhance T cell responses[1]. Another ligand for CD4 is the HIV-1 surface protein gp120. HIV-1 binds via gp120 to CD4 and the chemokine coreceptor, CCR5 or CXCR4, to trigger fusion of the virus with the cell membrane and mediate entry of HIV-1 into CD4+ T lymphocytes and monocyte-macrophages[2]. CD4 has a molecular mass of 55 kD and consists of an extracellular portion (residues 1–371), a transmembrane segment (372–395) and a cytoplasmic tail (396–433)[3]. The extracellular portion consists of four Ig-like domains[4–7], D1 to D4. Class II MHC binding extends over D1 and D2[1], whereas HIV-1 gp120 binds to D1[2].

The backbone of Ig domains are defined by β strands indicated by letters A through G. Following standard Ig nomenclature, one β sheet of the sandwich-like structure contains strands A, B and E, and the other sheet has strands C, C', F and G. The disulfide bond in D1 of CD4 is between Cys[16] in strand B and Cys[84] in strand F[4–6]. Similarly, the disulfide bond in D4 is between Cys[303] in strand B and Cys[345] in strand F[4,7]. In contrast, D2 has a truncated β barrel (75 residues compared to ~100 residues) and a nonstandard disulfide bond between Cys[130] in strand C and Cys[159] in strand F[4–6]. In effect, D2 has lost the cysteine in strand B and acquired one in strand C. This means that the disulfide bond in D2 is between strands in the same sheet rather than between sheets, as is more normal. The cysteine in strand C eliminates the normally conserved tryptophan in that position[4–6].

The geometry and strain of the D2 disulfide bond is also unusual. The disulfide is right- rather than left-handed and has a short (3.92 Å) $C_\alpha$-$C_\alpha$ distance compared to standard Ig disulfides[8] (6.6 Å) and right-handed disulfides[8] (5.07 Å). Calculation of the dihedral strain energies[8,9] of the disulfide bonds reveals that the D2 bond has a high strain energy (4.74 kcal mol$^{-1}$) compared to the D1 (2.28 kcal mol$^{-1}$) and D4

(1.71 kcal mol$^{-1}$) disulfide bonds. A high dihedral strain energy correlates with ease of reduction of the disulfide bond[8]. In addition, the D2 disulfide makes the least contribution to overall stability, as determined by enthalpy calculations[10]: D2 disulfide is 3.65 kcal mol$^{-1}$ compared to the D1 (4.36 kcal mol$^{-1}$) and D4 (3.97 kcal mol$^{-1}$) disulfides.

These features led us to investigate whether the D2 disulfide bond of CD4 was redox-active on the cell surface. We show here that the D2 disulfide bond can exist in the reduced dithiol form on the cell surface. Thioredoxin, a small protein reductant that is secreted by CD4+ T cells, appeared to regulate the redox state of the D2 disulfide. Reaction of the D2 and thioredoxin active-site dithiols with a peptide trivalent arsenical blocked entry of HIV-1 into susceptible cells. These findings indicate that reduction of the D2 disulfide bond played a role in HIV-1 entry. Perturbation of this redox event may be a worthwhile strategy for inhibiting HIV-1 infection.

## Results

### Cell-surface CD4 contains free thiol(s)

We labeled CD4+ cells with either sulfosuccinimidobiotin (SSB) or 3-(N-maleimidylpropionyl)biocytin (MPB). Both reagents are substantially membrane-impermeable[11,12]. SSB labels primary amines, whereas MPB will only label free thiols. Because there are no unpaired cysteines in the extracellular part of CD4, incorporation of MPB would indicate reduction of one or more of the three disulfide bonds. The thiol specificity of MPB was confirmed by labeling purified plasma proteins that lack or contain a free thiol. MPB labeled serum albumin, which contains a free thiol, but not prothrombin, which does not contain free thiols (data not shown).

Cell-surface CD4 incorporated both SSB and MPB (**Fig. 1a,b**). Labeling with MPB was thiol-specific, as preblocking of the MPB with

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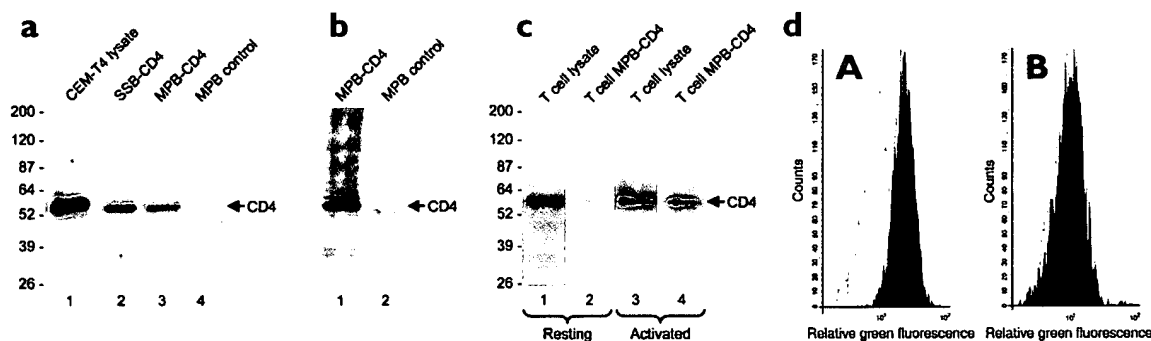
Figure 1. Cell-surface CD4 contains free thiol(s). (a) CEM-T4 cells were labeled with either SSB or MPB; the biotin-labeled proteins were collected and immunoblotted for CD4. Lane 1, CEM-T4 lysate (from 10⁶ cells); lane 2, SSB-labeled CEM-T4 CD4; lane 3, MPB-labeled CEM-T4 CD4. Biotin-labeled proteins were from 5 x 10⁶ cells. Lane 4, a control experiment in which MPB was preblocked with GSH before incubation with CEM-T4 cells. The positions of size markers, in kD, are shown. (b) CEM-T4 cells were labeled with MPB; CD4 was immunoprecipitated and blotted with peroxidase-streptavidin. Lane 1, MPB-labeled CD4 (from 7 x 10⁶ cells). Lane 2, a control experiment as in a. (c) Peripheral blood T cells or T cells activated with phytohemagglutinin for 3 days were labeled with MPB; the biotin-labeled proteins were collected and immunoblotted for CD4. Lanes 1 and 3, unactivated and activated T cell lysate (from 10⁶ cells), respectively. Lanes 2 and 4, MPB-labeled CD4 from unactivated and activated T cells (from 10⁷ cells), respectively. (d) gp120 bound equivalently to unlabeled or MPB-labeled cell-surface CD4. CEM-T4 cells were incubated without (filled histogram) or with (shaded histogram) MPB (A) or Leu3a mAb (B, filled histogram). FITC-gp120 was then added to the cells, and bound gp120 was measured by flow cytometry. The filled and shaded histograms in A overlap. The open histograms in A and B are controls, which used an irrelevant FITC-conjugated mAb.

reduced glutathione (GSH) ablated labeling. Comparison of the amount of SSB- versus MPB-labeled CD4 indicated that ~40% of CD4 on the surface of CEM-T4 cells contained one or more free thiols (Fig. 1c). A substantial fraction, therefore, of one or more of the three CD4 disulfide bonds could exist in the reduced form on the cell surface.

CD4 on the surface of human blood T cells (Fig. 1c), monocyte-macrophages (data not shown) and the human monocyte–macrophage line THP-1 (data not shown) also incorporated MPB. Activation of blood T cells with phytohemagglutinin for 3 days caused a ~45% increase in the fraction of total cell-surface CD4 that labeled with MPB (Fig. 1c). Another Ig fold receptor, Thy-1, was not labeled with MPB on the TIB-47 cell line (data not shown). These results indicated that CD4 on the surface of both T cells and macrophages was redox-active and that cell activation increased the reduction of CD4.

The effect of cell-surface CD4 labeling with MPB on HIV-1 gp120 binding to CD4 was measured by flow cytometry. CEM-T4 cells were labeled with MPB and then incubated with fluorescein isothiocyanate (FITC)-conjugated gp120. Treatment of the T cells with MPB had no effect on binding of gp120 (Fig. 1dA). gp120 bound specifically to cell-surface CD4, as preblocking CD4 with the monoclonal antibody (mAb) Leu3a ablated binding (Fig 1dB). Reduction of CD4, therefore, did not significantly affect binding of gp120 to D1 of CD4.

## The D2 disulfide of CD4 is redox-active

These labeling experiments showed that one or more of the three disulfide bonds in cell-surface CD4 could exist in the reduced dithiol form. To determine which of the disulfide bonds was redox-active, we stably transfected disulfide bond mutants of CD4 into cultured cells and tested for the presence of free thiols by labeling with MPB. We prepared cysteine→alanine mutants of the three pairs of cysteine residues in D1, D2 or D4 of CD4. Several attempts at stably transfecting the human CD4⁻ T cell line A2.01 with the CD4 disulfide bond mutants in the eukaryotic expression vector SRα[13] were unsuccessful (data not shown). Stable transfection of the equivalent cysteine→serine mutants into A2.01 cells was also unsuccessful (data not shown). In contrast, wild-type CD4 was expressed on the surface of A2.01 cells (data not shown). These findings suggested that the disulfide bond mutants were recognized by the T cells as misfolded and targeted for degradation. Transfection and surface expression of the CD4 disulfide bond mutants could be achieved, however, in human fibrosarcoma cells.

Surface expression of wild-type CD4 and the D2 disulfide mutant in HT1080 cells is shown (Fig. 2a). Immunoblotting of the wild-type cell lysate indicated the presence of both monomers and dimers of CD4 (Fig. 2b). The CD4 dimers resolved into monomers upon reduction and alkylation of the lysate, which indicated that the dimers were



Figure 2. The CD4 D2 disulfide is redox-active. (a) Human fibrosarcoma cells expressing either wild-type (WT) CD4, or a mutant CD4 in which both D2 cysteines are mutated to alanines, were incubated with a control FITC-conjugated mAb (open histograms) or with FITC-conjugated Leu3a mAb (filled histograms); the bound antibodies were measured by flow cytometry. (b) The HT1080 CD4 WT (lane 1) and CD4 D2 mutant (lane 2) cells were lysed, and lysate from 2 x 10⁵ cells was immunoblotted for CD4. M, CD4 monomer; D, CD4 dimer. (c) The HT1080 CD4 WT (lane 1) and D2 (lane 2), D1 (lane 3) and D4 (lane 4) mutant cells were labeled with MPB and the biotin-labeled proteins collected and immunoblotted for CD4. MPB-labeled proteins were from 7 x 10⁶ cells. The positions of size markers, in kD, are shown.
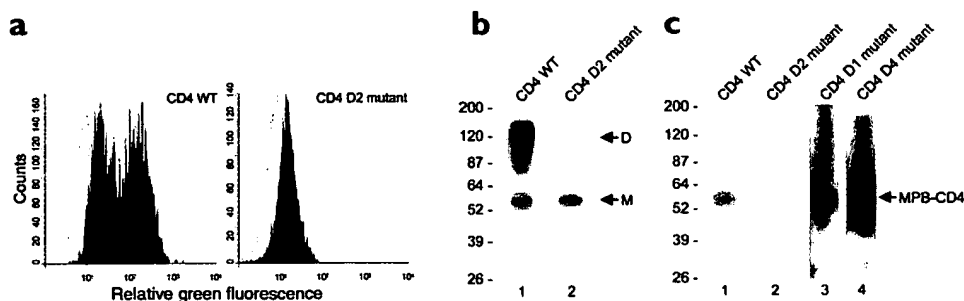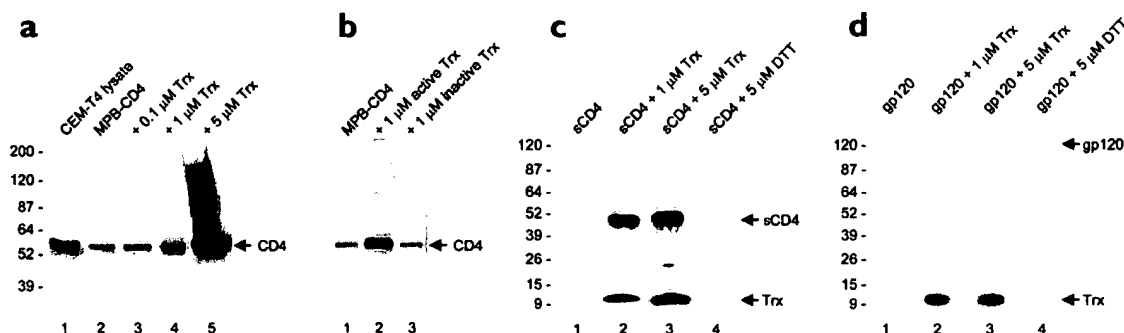
**Figure 3. Reduction of cell-surface and soluble CD4 by thioredoxin.** (a) CEM-T4 cells were incubated without (lane 2) or with increasing concentrations of human thioredoxin (Trx) (lanes 3–5), labeled with MPB and the biotin-labeled proteins collected and immunoblotted for CD4. Lane 1, CEM-T4 lysate. The positions of size markers, in kD, are shown. (b) CEM-T4 cells were incubated with either redox-active thioredoxin mutant (lane 2) or redox-inactive thioredoxin mutant (lane 3), labeled with MPB and the biotin-labeled proteins were collected and immunoblotted for CD4. Lane 1, untreated CEM-T4 cells. (c) sCD4 was incubated without (lane 1) or with (lanes 2 and 3) thioredoxin or dithiothreitol (lane 4) and then labeled with MPB and blotted with peroxidase-streptavidin. MPB-labeled sCD4 and thioredoxin are shown. (d) gp120 was incubated without (lane 1) or with (lanes 2 and 3) thioredoxin or dithiothreitol (lane 4), then labeled with MPB and blotted with peroxidase-streptavidin. MPB-labeled thioredoxin is shown.

disulfide-bonded (data not shown). The extent of dimer formation varied with each experiment, but was always apparent. CD4 D1 and D4 disulfide mutants also formed dimers (data not shown). In contrast, the CD4 D2 disulfide mutant did not form dimers (Fig. 2b). Labeling of wild-type and the disulfide mutant CD4s on the surface of HT1080 cells with MPB is shown (Fig. 2c). The monomeric forms of wild-type CD4 and the D1 and D4 disulfide bond mutants incorporated MPB, whereas the D2 disulfide mutant was not labeled, despite similar cell-surface expression of the monomeric forms of the wild-type and mutant CD4s (data not shown). These results indicated that the D2 disulfide of CD4 could exist in the reduced dithiol form on the cell surface and that it was involved in thiol-dependent dimerization of CD4.

**Reduction of CD4 by thioredoxin**

The finding that blood T cell activation resulted in reduction of the D2 disulfide in cell-surface CD4 suggested that the oxidation state of the D2 disulfide bond was controlled by the cell (Fig. 1c). This may be accomplished by the secretion of a disulfide bond reductase by the CD4+ T cell. Incubation of CEM-T4 cells with increasing concentrations of the protein reductant thioredoxin resulted in increasing disulfide reduction of cell-surface CD4 (Fig. 3a). The redox properties of thioredoxin were required for reduction of cell-surface CD4, as a redox-inactive thioredoxin mutant[14] did not reduce CD4 (Fig. 3b). Incubation of CEM-T4 cells with the same concentrations of another disulfide bond reductase, protein disulfide isomerase, also failed to increase labeling of CD4 with MPB (data not shown).

Soluble CD4 containing the four Ig-like domains (sCD4)[15] was tested for disulfide reduction by thioredoxin. Thioredoxin-facilitated reduction of sCD4 and incorporation of MPB is shown (Fig. 3c). Disulfide reduction was selective for thioredoxin because equivalent concentrations of the strong reductant dithiothreitol (Fig. 3c) or protein disulfide isomerase (data not shown) did not reduce sCD4. Soluble gp120 could not be reduced by thioredoxin or dithiothreitol (Fig. 3d). Reduction of the CD4 D2 disulfide, therefore, could occur independent of the cell surface and is selective for thioredoxin.

**Labeling of CD4 with trivalent arsenic**

The proximity of the two free thiols generated in D2 by reduction of the disulfide bond was examined with the chemistry of trivalent arsenic. Trivalent arsenicals form high-affinity ring structures with closely spaced dithiols, but react very poorly with monothiols or dithiols that are not in close proximity[16–18]. We attached the trivalent arsenical phenylarsenoxide to the thiol of reduced glutathione to produce 4-(N-(S-glutathionylacetyl)amino)phenylarsenoxide (GSAO)[18]. GSAO is substantially membrane-impermeable and binds tightly to synthetic and peptide dithiols and the thioredoxin active-site dithiol, but not to monothiols[18]. To detect incorporation of GSAO into CD4, a biotin moiety was attached through a spacer arm to the primary amino group of the γ-glutamyl residue of GSAO (GSAO-B)[18]. Cell-surface CD4 incorporated GSAO-B (Fig. 4). The labeling of CD4 with GSAO-B was specific, as a fourfold molar excess of the small synthetic dithiol dimercaptopropanol competed with GSAO-B for labeling. As the CD4 D2 thiols were sufficiently close to complex with a trivalent arsenical, they were predicted to be close enough to reoxidize to the disulfide bond. This result indicated that reduction of the D2 disulfide bond was likely to be reversible.



**Figure 4. Labeling of cell-surface CD4 with GSAO-B.** CEM-T4 were labeled with either MPB (lane 1) or GSAO-B (lane 2); the biotin-labeled proteins were collected and immunoblotted for CD4. Lane 3, a control experiment in which GSAO-B was incubated with CEM-T4 cells in the presence of a fourfold molar excess of 2,3-dimercaptopropanol. Biotin-labeled proteins were from 2 × 10⁴ cells. The positions of size markers, in kD, are shown.

**Inhibition of HIV-1 entry by GSAO**

The requirement for redox changes in CD4 for HIV-1 entry into susceptible cells was evaluated by measuring viral DNA accumulation after a high multiplicity HIV-1_HXB2 infection of HuT-78 T cells in the presence of GSAO[19]. Cells were preincubated with GSAO or the corresponding pentavalent arsenical 4-(N-(S-glutathionylacetyl)amino) phenylarsonic acid (GSAA)[18], before infection with HIV-1_HXB2. GSAA controls for the dithiol-reactivity of GSAO, as pentavalent arsenicals do not react with thiols. The reverse transcriptase inhibitor lamivudine (3TC), which inhibits HIV-1 DNA synthesis after viral entry, was used as a positive control. Infected cells were washed and then treated with trypsin to minimize any cell surface–associated virus and then cultured
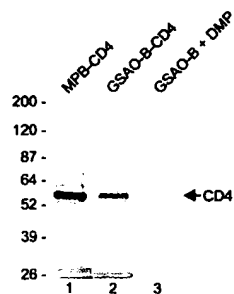
**Figure 5. Inhibition of HIV-1 entry into CD4⁺ cells by GSAO.** (a) Effect of 100 µM GSAO or GSAA, or 10 µM of the reverse transcriptase inhibitor 3TC, on the accumulation of HIV-1 DNA 8 h after infection of HuT-78 cells with HIV-1$_{IOXB2}$. Quantitative PCR amplification of gag DNA sequences (upper panel) was done on cell-equivalent amounts of extrachromosomal DNA (lower panel) from duplicate cultures. The HIV-1 DNA standards used were known cell-equivalents of chromosomal DNA isolated from a mixture of three persistently infected cell lines. (b) Effect of GSAO on entry of a CD4-dependent or -independent reporter virus into CD4⁺ or CD4⁻ cells. Pseudotyped NL4-3-Luc.R⁻E⁻



virus containing Env that confers dual tropism (89.6) or a mutant Env that uses CXCR4 for entry and is unable to bind CD4 (8xD368R) were incubated with CD4⁺ (A3.01) or CD4⁻ (CXCR4⁺293T) cells, respectively, in the absence or presence of 100 µM GSAO for 1 h. Cells were cultured in the absence or presence of 100 µM GSAO for 24 or 72 h and analyzed for luciferase expression. Data are mean ± range from duplicate experiments. (c) Concentration dependence of GSAO. Pseudotyped NL4-3-Luc.R⁻E⁻ virus containing a dual-tropic Env (89.6) or a fusion defective Env mutant (89.6W610F) was incubated with CD4⁺ A3.01 T cells in the absence or presence of 10–100 µM GSAO or GSAA for 1 h. Cells were cultured in the absence or presence of 10–100 µM GSAO or GSAA for 24 h and analyzed for luciferase expression. Data are mean ± range of duplicate experiments.
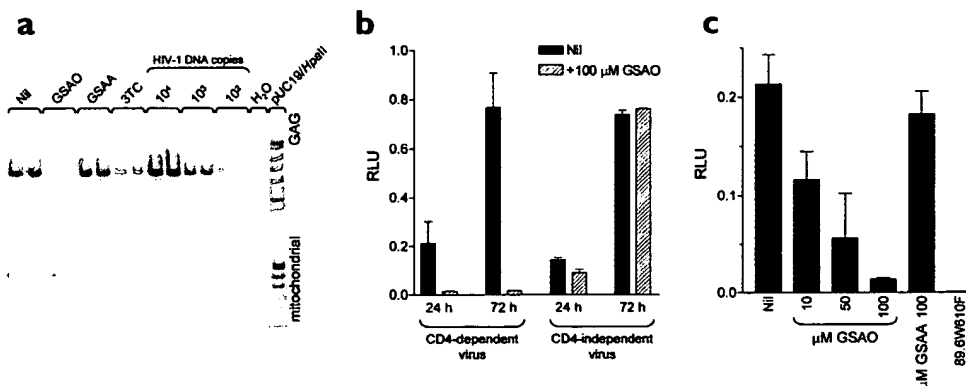
for 8 h. Viral gag DNA was then detected by quantitative polymerase chain reaction (PCR). Reverse-transcribed viral DNA was evident in Nil- (as in untreated) and GSAA-treated cultures, but was absent or substantially reduced in cultures treated with GSAO or 3TC, respectively (Fig. 5a). Neither GSAO nor GSAA had any effect on cell viability (data not shown). These results show that GSAO inhibited HIV-1 infection of HuT-78 T cells at a stage before reverse transcription of the viral genome, which is consistent with entry-level inhibition.

To test the requirement of CD4 for the inhibitory effects of GSAO on HIV-1 entry, inhibition of CD4-dependent or -independent reporter virus entry into CD4⁺ or CD4⁻ cells by GSAO was measured. GSAO (100 µM) effectively blocked the entry of a dual-tropic[20] (HIV-1$_{89.6}$) reporter virus into CD4⁺ A3.01 T cells, but had no significant effect on entry of a reporter virus that uses CXCR4 for entry (HIV-1$_{8xD368R}$)[21] into CXCR4⁺CD4⁻ 293T cells (Fig. 5b). GSAA (100 µM) had no effect on either CD4-dependent or -independent virus entry (data not shown). This result also controlled for possible effects of GSAO on events after viral entry and before expression of the viral DNA. The half-maximal inhibitory concentration of GSAO in this system was ~10 µM (Fig. 5c). HIV-1 entry measured by this system was specific, as a fusion defective mutant of HIV-1$_{89.6}$(89.6W610F) (data not shown) did not enter CD4⁺ cells (Fig. 5c).

These observations indicated that locking the CD4 and the thioredoxin active-site dithiols in the reduced state with a trivalent arsenical blocked entry of HIV-1 into CD4⁺ cells.

## Discussion

The CD4 D2 disulfide bond is unusual because it links cysteines within the one β-sheet rather than across a barrel and is right- rather than left-handed. It was not surprising, therefore, that the bond is particularly strained in comparison to the D1 and D4 disulfide bonds. The more strain on a disulfide bond, the more readily it is reduced[8]. These features of the D2 disulfide bond suggest that it could be reduced on the surface of cells. Indirect support for this theory was provided by the finding that thiol-oxidizing reagents such as HgCl₂[22] and 5,5'-dithiobis(2-nitrobenzoic acid)[23] facilitate dimerization and oligomerization of CD4 on the cell surface and that uptake of HIV-1 by CD4⁺ T cells is inhibited by membrane-impermeable thiol-reactive reagents[24]. Using MPB, we showed here that

the D2 disulfide of CD4 is redox-active. HIV-1 entry into T cells was dependent on the redox state of CD4.

Disulfides that bridge across the nearest neighbor positions in antiparallel β-strands are uncommon[25], although crystal structures of model cysteine peptides[26] and antiparallel dimers[27] have demonstrated that disulfide bridging between strands in antiparallel β-sheets can occur. Side-by-side disulfide bonds have been predicted in other members of the Ig superfamily[28]. The Ig domain of the α chain of CD8, a receptor with a similar function to CD4, also has an unconventional disulfide linkage[29]. There are three cysteines in this Ig domain: one each in stands B and F, as usual, and a third that is two residues before the conserved tryptophan in strand C. The disulfide in this domain links this extra cysteine in strand C with the cysteine in strand B, leaving the cysteine in stand F unpaired[29]. The disulfide bond in Ig folds is usually between the cysteines in strands B and F.

Reduction of disulfide bonds is usually facilitated by another redox protein. It is likely, therefore, that reduction of cell-surface CD4 was facilitated by a cell-derived factor. Thioredoxin is a 12-kD redox protein[30] that is secreted by CD4⁺ T cells and binds to their surface[31–34]. Indeed, we showed that thioredoxin reduced cell-surface and soluble CD4, but not soluble gp120. CD4⁺ T cell activation is associated with increased secretion of thioredoxin[31,33], which is consistent with the increased reduction of peripheral blood T cell–surface CD4 upon phytohemagglutinin activation. It appears, therefore, that the redox state of cell-surface CD4 is controlled by thioredoxin secreted by the same or other cells.

The question of whether the D2 disulfide can exchange between oxidized and reduced forms is an important one for CD4 function. Disulfide exchange in D2 is supported by the link between cell activation and reduction of cell-surface CD4. Reoxidation of the dithiol would imply that reduction of the disulfide did not result in significant separation of the thiols. The spacing of the D2 thiols was examined with a hydrophilic trivalent arsenical. Trivalent arsenicals react with thiols that are within ~10 Å of each other to form stable cyclic dithioarsinites[16], but react very poorly with monothiols or dithiols that are not in close proximity[18]. For instance, the hydrophilic trivalent arsenical GSAO binds tightly to the active site dithiol of thioredoxin but does not bind to the single free thiol of albumin[18]. GSAO also bound to

the reduced dithiol form of CD4. This result implied that the two thiols of reduced CD4 were in close proximity, and—by analogy with the dithiol of thioredoxin—are predicted to be close enough to reoxidize to the disulfide bond. Dynamic exchange of the D2 disulfide–dithiol, therefore, may regulate CD4 structure and function.

These findings suggested that the oxidized and reduced forms of CD4 exist on the cell surface in equilibrium and are controlled by thioredoxin secreted by the T cell. We propose that reduction of the D2 disulfide by thioredoxin will result in oxidation of the dithiol active-site of thioredoxin. For thioredoxin to catalyze reduction of another CD4 molecule, a mechanism is required to reduce the oxidized form of the protein. This may be accomplished by thioredoxin reductase, which is secreted by peripheral blood mononuclear cells and is present in plasma at a concentration of 18 ng ml$^{-1}$ (ref. 35). It is important to note, however, that our findings do not exclude the possibility that thioredoxin-mediated exchange of the D2 disulfide may occur indirectly through redox control of another cell-surface protein.

Perturbation of the redox chemistry of the D2 dithiol and thioredoxin by the hydrophilic trivalent arsenical blocked HIV-1 entry into susceptible cells in a CD4-dependent manner. The process of HIV-1 infection begins with binding of the viral envelope glycoprotein, gp120, to both the D1 domain of CD4 and either CCR5 or CXCR4. Infection is complete once the virus fuses with the cell membrane[2]. The reason why redox activity of the D2 disulfide is important for HIV-1 infection is unknown. The viral envelope protein gp120 bound equivalently to unlabeled or GSAO- or MPB-labeled cell-surface CD4. This finding implied that redox changes in the D2 disulfide bond were not important for initial binding of HIV-1 but rather for post-binding events before fusion.

It is likely that disulfide exchange in D2 is involved in conformational changes in CD4 after binding of HIV-1[36,37]. For instance, a mAb that recognizes D2 blocks post-binding events relevant to plasma membrane-virus envelope fusion without affecting the interaction of virions with CD4[38,39]. It is also possible that disulfide-dependent self-association of CD4 through the D2 domain plays a role in HIV-1 entry[23].

Notably, plasma concentrations of thioredoxin in HIV-1–infected individuals are inversely correlated with CD4$^+$ cell numbers[40]. In addition, survival of HIV-1–infected individuals with chronically increased plasma thioredoxin concentrations and <200 CD4$^+$ cells per µl is significantly impaired relative to infected individuals with >200 CD4$^+$ cells per µl[41]. Also, the thiol-reactive compounds N-acetyl-L-cysteine[42,43], cystamine[44,45], cysteamine[46] and D-penicillamine[47,48] all have anti–HIV-1 activity. It may be that the efficacy of these drugs are, in part, due to formation of mixed disulfides with the dithiol forms of thioredoxin and/or CD4.

Despite the success in developing regimens that block HIV-1 transcription and assembly, problems of drug resistance, latent viral reservoirs and drug toxicity highlight the need for drugs with different modes of action. In particular, compounds that block HIV-1 entry would complement those that interfere with viral replication[2,49]. The redox changes in D2 we describe here represent a new target for HIV-1 entry inhibitors. Dithiol-reactive compounds such as GSAO are one way of blocking this redox chemistry.

## Methods

**Labeling of CD4$^+$ cells or sCD4.** Peripheral blood mononuclear cells were prepared from fresh citrated blood by Ficoll-Hypaque density gradient separation. Monocytes were separated from the T cells by allowing them to attach to tissue-culture plastic overnight. The unattached T cells were depleted of CD8$^+$ cells with antibody-conjugated magnetic microbeads. Blood T cells or CEM-T4 cells (NIH AIDS Research and Reference Reagent Program), 1 ml of 5 × 10$^5$–10 × 10$^6$ cells ml$^{-1}$ in Hank's balanced salt solution, were incubated with SSB (Pierce, Rockford, IL), MPB (Molecular Probes, Eugene, OR) or GSAO-B (100 µM) for 30

min at room temperature. On one occasion, MPB was preblocked with reduced glutathione (GSH) before incubation with cells or cells were labeled with GSAO-B in the presence of 2,3-dimercaptopropanol (400 µM, Fluka, Sydney, Australia). On another occasion, cells were preincubated with thioredoxin or dithiothreitol for 1 h at 37 °C, then labeled with MPB. The thioredoxin active site cysteines, residues 32 and 35, were replaced by serines in the redox-inactive mutant. Another pair of conserved cysteines, residues 69 and 73, were replaced by serines in the redox-active mutant[14]. Unreacted SSB was quenched with glycine (200 µM), whereas unreacted MPB was quenched with GSH (200 µM) for 30 min at room temperature. The labeled cells were washed three times with PBS and sonicated in 1 ml of ice-cold buffer (50 mM Tris HCl and 0.15 M NaCl at pH 8.0) containing 1% Triton X-100, 10 µM leupeptin, 10 µM aprotinin, 2 mM phenylmethylsulfonyl fluoride and 5 mM EDTA. Streptavidin-agarose beads (50 µl of packed beads, Sigma-Aldrich, Sydney, Australia) were incubated with the cell lysates for 1 h at 4 °C on a rotating wheel to isolate the biotin-labeled proteins. The streptavidin-agarose beads were washed five times with buffer (50 mM Tris HCl and 0.15 M NaCl at pH 8.0) containing 0.05% Triton X-100, and the biotin-labeled proteins were released from the beads by boiling in 30 µl of SDS-Laemmli buffer for 2 min. Samples were resolved on 4–15% SDS-PAGE under nonreducing conditions and transferred to polyvinylidene difluoride (PVDF) membrane. Proteins were detected by immunoblot with 5 µg ml$^{-1}$ of anti–murine CD4 (mAb Leu3a, Becton Dickinson, Bedford, MA) and a 1:2000 dilution of horseradish peroxidase (referred to as peroxidase throughout)–rabbit anti-mouse (Dako, Carpinteria, CA). Chemiluminescence films were analyzed with a GS-700 Imaging Densitometer and Multi-Analyst software (BioRad, Hercules, CA).

sCD4 and HIV-1$_{SF2}$ gp120 were from the NIH AIDS Research and Reference Reagent Program. sCD4 or gp120 (10 µg ml$^{-1}$) was incubated without or with thioredoxin or dithiothreitol for 10 min at 37 °C and then labeled with MPB (100 µM) for 30 min at room temperature. The unreacted MPB was quenched with GSH (200 µM) for 30 min at room temperature and the sCD4 or gp120 (0.1 µg) was resolved on SDS-PAGE and blotted with streptavidin peroxidase to detect the biotin label.

**Immunoprecipitation of CD4.** CEM-T4 cells were labeled with MPB, incubated with Leu3a mAb (5 µg ml$^{-1}$) for 30 min, washed three times and lysed in 0.5 ml of buffer (50 mM Tris HCl, 0.15 M NaCl at pH 8.0) containing 0.5% Triton X-100, 0.05% Tween 20, 100 µg ml$^{-1}$ bacitracin, 2 mM benzamidine and 2 mM phenylmethylsulfonyl fluoride for 30 min at 4 °C. The detergent-insoluble material was removed by centrifugation at 12000g for 30 min, and the supernatant was incubated with 10$^7$ goat anti–mouse IgG–coated Dynabeads (Dynal, Melbourne, Australia) for 60 min. All incubations were at 4 °C. The beads were washed and the bound CD4 released by boiling the beads in 30 µl of SDS-Laemmli buffer for 2 min. Samples were resolved on 4–15% SDS-PAGE under nonreducing conditions and transferred to PVDF membrane. MPB-labeled proteins were blotted with peroxidase-streptavidin (Amersham, Sydney, Australia) and used at a 1:1000 dilution.

**gp120 flow cytometry assay.** HIV-1$_{SF2}$ gp120 was labeled with FITC (Molecular Probes), according to the manufacturer's instructions. CEM-T4 cells (10$^6$ cells ml$^{-1}$) were incubated without or with MPB (100 µM) for 30 min at 37 °C in serum-free media. On one occasion, cells were incubated with Leu3a mAb (20 µg ml$^{-1}$) for 30 min at 4 °C in serum-free media. FITC-gp120 (10 nM final concentration) was then added to the cells and incubated for 1 h at 37 °C. The cells were washed twice with PBS that contained 2% fetal bovine serum, and binding of gp120 to the cells was measured by flow cytometry with a FACStar Plus cytometer (Becton Dickinson). Ten-thousand cells were acquired at a flow rate of 500–1000 particles per second.

**Mutation of CD4.** The T4-pMV7 plasmid, which contains full-length human CD4 cDNA, was from the NIH AIDS Research and Reference Reagent Program. The mammalian expression vector pcDNA3, which contains a CMV promoter, was from Invitrogen (San Diego, CA). A 3-kb CD4 cDNA was extracted from the T4-pMV7 plasmid as an EcoRI fragment and subcloned into the pcDNA3 vector to produce pcDNA3/CD4 wild-type. The pairs of cysteine residues, which constitute the disulfide bonds in D1 (Cys$^{16}$ and Cys$^{84}$), D2 (Cys$^{130}$ and Cys$^{159}$) and D4 (Cys$^{303}$ and Cys$^{345}$), were individually mutated to alanine or serine residues. The first cysteine in each domain was mutated to alanine or serine with the QuikChange Site-Directed Mutagenesis Kit (Integrated Sciences, La Jolla, CA). The single cysteine mutants were then used as templates to mutate the other cysteine. All mutations were confirmed by automatic sequencing (ABI-377 Automatic Sequencer, Applied Biosystems, Foster City, CA). Human fibrosarcoma HT1080 cells (ATCC, Rockville, MD) (10$^5$ cells) were transfected with pcDNA/CD4 vectors (0.5–10 µg) with Fugene 6 (3–15 µl, Boehringer, Mannheim, Germany). Stably transfected cells were selected by incubation with medium containing 400 µg ml$^{-1}$ of G418 (Life Technologies, Paisley, UK). Surface expression of CD4 was measured by flow cytometry with FITC-conjugated Leu3a mAb and a FACStar Plus cytometer (Becton Dickinson). Ten-thousand cells were acquired at a flow rate of 500–1000 particles per second.

**gag PCR assays.** HIV-1$_{IIIB}$ infection of the CD4$^+$ T lymphoblastoid cell line HuT-78 was done as described[19]. Briefly, virus inoculum was obtained by high-density culture of the persistently HTLV-IIIB (HIV-1$_{IIIB}$)-infected H3B cell line[50] and was determined to have a TCID$_{50}$ of 3.16 × 10$^4$ U ml$^{-1}$. Subcultured HuT-78 cells were infected at an MOI of 1 with a centrifugal enhancement infection protocol[19]. GSAO, GSAA or 3TC were preincubated with cells for 45 min and then washed once in culture media before infection. After centrifugation with virus, cells were washed once in serum-free media, treated with 0.05% trypsin for 5 min at 37 °C to minimize trapped virus and then washed twice in culture medium before plating

in a 48-well tray at a density of $10^6$ cells ml$^{-1}$ and culturing for 8 h. Mitochondrial PCRs were done with primers M1 (5'-GACGTTAGGTCAAGGTGTAG-3') and M2 (5'-GGTTGTCTG GTAGTAAGGTG-3') on ~50 cell-equivalents of Hirt supernatant DNA[19]. The *gag* PCRs were with primers GAG-P1(+) (5'-GAGGAAGCTGCAGAATGGG-3') and GAG-III(-) (5'-CTGTGAAGCTTGCTCGGCTC-3') on ~1000 cell-equivalents of Hirt supernatant DNA[19]. Southern hybridizations were done with [$\alpha$-$^{32}$P]dATP-labeled fragments corresponding to nt 1320–1715 of the human mitochondrial DNA sequence and to nt 1408–1722 of the HIV-1$_{HXB2}$ sequence. The HIV-1 DNA copy number standard used was a mixture of equivalent amounts of chromosomal DNA extracted from known numbers of H3B, ACH-2 and 8E5 persistently infected cells containing two, one and one copies of integrated HIV-1 DNA, respectively[19].

**Pseudotyped reporter virus assays.** Pseudotyped NL4-3-Luc.R$^-$E$^-$ virus stocks were generated by transfecting 293T cells with pNL4-3.Luc.R$^-$E$^-$ (NIH AIDS Research and Reference Reagent Program) and a pcDNA3.1 expression plasmid containing HIV-1 *Env* from either HIV-1$_{89.6}$ (dual-tropic)[20], HIV-1$_{89.6N163F}$ (a fusion defective mutant of 89.6)[20] or HIV-1$_{xLDV4RR}$ (a mutant variant of HIV-1$_{IIIB}$ that uses CXCR4 for entry and is unable to bind CD4)[21]. All transfections were with 1 $\mu$g of DNA and Fugene 6 transfection reagent (Roche Diagnostics, Mannheim, Germany). Virus-containing supernatants were collected 72 h after transfection, filtered and frozen at $-80$ °C.

CD4$^+$ A3.01 T cells (1 × 10$^6$ cells in 1 ml) or 293T cells (0.25 × 10$^6$ cells in 1 ml) transfected with pc.FUSIN (NIH AIDS Research and Reference Reagent Program) were preincubated with 10–100 $\mu$M GSAO or GSAA for 30 min at 37 °C in 12-well plates in serum-free media. Reporter virus (0.5 ml) was then added for 1 h at 37 °C. Cells were washed twice in serum-free media and then treated with 0.05% trypsin for 5 min at 37 °C to minimize trapped virus[19]. Cells were washed another two times in serum-containing media and incubated in 12-well plates in complete media containing 10–100 $\mu$M GSAO or GSAA for 24 or 72 h. Luciferase activity was measured using the Promega luciferase assay system (Madison, WI) and expressed as relative light units (RLU).

**Competing interests statement**
The authors declare that they have no competing financial interests.

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# Disulfide exchange in domain 2 of CD4 is required for entry of HIV-1

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In the AOP version of this article some text was incorrect. On page 3, column 2, line 6 from the bottom the sentence should read "GSAA controls for the dithiol-reactivity of GSAO, as pentavalent arsenicals do not react with thiols." On page 6, column 1, line 5, a closing parenthical mark, rather than the second prime mark, should appear at the end of the GAG-III (–) sequence. In Figure 3a and b, the arrows to the right of the immunoblot panels, which show the position of CD4, should be labeled "CD4", not "sCD4". These errors have been corrected in the HTML version and will appear correctly in print. The PDF version available online has been appended.

# Protein-disulfide Isomerase-mediated Reduction of Two Disulfide Bonds of HIV Envelope Glycoprotein 120 Occurs Post-CXCR4 Binding and Is Required for Fusion*

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The human immunodeficiency virus (HIV) envelope (Env) glycoprotein (gp) 120 is a highly disulfide-bonded molecule that attaches HIV to the lymphocyte surface receptors CD4 and CXCR4. Conformation changes within gp120 result from binding and trigger HIV/cell fusion. Inhibition of lymphocyte surface-associated protein-disulfide isomerase (PDI) blocks HIV/cell fusion, suggesting that redox changes within Env are required. Using a sensitive assay based on a thiol reagent, we show that (i) the thiol content of gp120, either secreted by mammalian cells or bound to a lymphocyte surface enabling CD4 but not CXCR4 binding, was 0.5–1 pmol SH/pmol gp120 (SH/gp120), whereas that of gp120 after its interaction with a surface enabling both CD4 and CXCR4 binding was raised to 4 SH/gp120; (ii) PDI inhibitors prevented this change; and (iii) gp120 displaying 2 SH/gp120 exhibited CD4 but not CXCR4 binding capacity. In addition, PDI inhibition did not impair gp120 binding to receptors. We conclude that on average two of the nine disulfides of gp120 are reduced during interaction with the lymphocyte surface after CXCR4 binding prior to fusion and that cell surface PDI catalyzes this process. Disulfide bond restructuring within Env may constitute the molecular basis of the post-receptor binding conformational changes that induce fusion competence.

The mature human immunodeficiency virus (HIV)[1] envelope (Env) is composed of the surface glycoprotein (gp) 120 and transmembrane gp41 subunits (1). HIV binding to CD4^{+} lymphocytes is initiated by gp120 interaction with cell surface CD4, which creates a high affinity binding site on gp120 for the coreceptor CXCR4. Interactions between gp120 and the receptors lead to structural changes within Env that eventually promote the unmasking of the fusion peptide present on gp41, its insertion into the target cell surface and HIV/cell membrane fusion (2–6).

Although structural changes within Env following interaction with the cellular receptors are mostly attributed to the intrinsic properties of the viral envelope, they occur in the context of proteolytic and other catalytic cell surface activities that are indispensable for membrane fusion to occur (7–9). One such activity is cell surface protein-disulfide isomerase (PDI), which has been implicated in the process of HIV infection (9, 10).

PDI is a member of the thioredoxin superfamily. It catalyzes reduction, oxidation, and thiol-disulfide interchange reactions and has important roles in the folding of secretory proteins in the biosynthetic pathway (11, 12). On the surface of the cell, it has been shown to cause structural modifications of exofacial proteins involved in biological process (11, 13–15). Clustered on the lymphocyte surface in the vicinity of CD4-enriched regions (9), PDI may influence the conformation modifications that occur during the interaction of HIV Env with the target cell surface receptors through a partial reorganization of the network of the disulfide bonds of the viral protein. Alternatively, it may influence the thiol-disulfide content of other cell surface antigens involved in the HIV/cell fusion process. Experimental data support both possibilities because PDI inhibition interferes with the virus/lymphocyte fusion process post-CD4 binding (9).

Besides the observation that PDI inhibition blocks the HIV-replicative cycle (9, 10), the plausible involvement of redox changes of Env as part of the HIV-lymphocyte interaction is suggested by the observation that an unusually dense cluster of disulfide bonds occurs close to the receptor binding surfaces (16). As the precise molecular basis of the Env conformational changes that take place upon fusion remains enigmatic, we undertook a detailed study of the relationship between cell surface-associated receptor binding and the thiol-disulfide content of HIV Env. We focused solely on the redox state of the surface protein gp120 as it contains 9 of the 10 disulfide bonds of Env$_{Lai}$ (17).

Here, using a sensitive thiol reagent, we show that on average two Env disulfide bonds are reduced during interaction with the lymphocyte surface immediately prior to fusion. We provide evidence that a reductase activity belonging to the PDI family is involved. We propose that disulfide bond restructuring constitutes the molecular basis of the post-receptor binding conformational changes that induce fusion competence.

## EXPERIMENTAL PROCEDURES

*Reagents*—The impermeant thiol reagent 3-(N-maleimidylpropionyl)-biocytin (MPB) was purchased from Molecular Probes (Eugene, OR). Reagents including thyroglobulin (Tg), the PDI inhibitors 3,3′,5 triiodothyronine (T3) (18) and bacitracin (10) were purchased from Sigma. Env$_{GB8}$ and Env$_{Lai}$ (reference batches) were supplied by the EVA Medical Research Council AIDS Reagent Program (Potters Bar, United

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[1] The abbreviations used are: HIV, human immunodeficiency virus; Env, envelope; gp, glycoprotein; PDI, protein-disulfide isomerase; MPB, 3-(N-maleimidylpropionyl)biocytin; Tg, thyroglobulin; VV, vaccinia virus; CD4^{+} lymphoid cells; PBS, phosphate-buffered saline; MES, 4-morpholineethanesulfonic acid; SDF, stromal-derived factor; sCD4, soluble CD4; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid.

Kingdom) and the ANRS (Paris, France), respectively. Purified recombinant soluble CD4 (sCD4) was supplied by the EVA Medical Research Council AIDS Reagent Program and was labeled (30 $\mu$Ci/$\mu$g) using iodogen before purification by Sepharose $G_{25}$ chromatography as described previously (9). SDF1-$\alpha$ was purchased from Peprotech (London, United Kingdom) and was labeled (150 $\mu$Ci/$\mu$g) using lactoperoxidase before purification by Sepharose $G_{25}$ chromatography as described previously (19, 20). Sheep polyclonal antibody D7324 (Aalto, Dublin, Ireland) (19) is an anti-peptide antibody directed against the C terminus of gp120 (APTKAKRRVVQREKR sequence). Rabbit polyclonal antibody SPA-890 (Stressgen) (15) is directed against bovine PDI. Vaccinia virus (VV) 9-1 (kindly provided by M. P. Kieny) (21) and VBD3 (kindly provided by R. Collman and R. Doms through the National Institutes of Health AIDS Program) (22) are VV vectors encoding the native form of $Env_{Lai}$ and $Env_{89-6}$, respectively. Following secretion into the cell supernatant, the viral antigens were concentrated and processed for binding experiments as described in previously (19).

*Cell Infections and Env Production*—Human CD4$^+$ lymphoid cells (CEM) ($10^6$ cells/ml) and CD4$^-$ baby hamster kidney 21 cells ($10^6$ cells/ml) were cultured and infected as described previously (9, 23). For Env expression, cells were infected using VV vectors (CEM cells: 3–5 plaque-forming units/cell; baby hamster kidney 21 cells: 5–10 plaque-forming units/cell) in serum-free medium to enable further supernatant concentration (30 times) using the Ultrafree 15 device system (30-kDa cutoff; Millipore, St Quentin en Yvelines, France).

## MPB Labeling

*Thiol Content of Purified Antigens*—Samples containing either Tg or Env diluted in phosphate-buffered saline (PBS), pH 7.4, were dot-blotted onto a nitrocellulose filter (Schleicher & Schuell). After blocking with PBS, 2% casein, filters were incubated with MPB (0.1 mM, 30 min at 25 °C). After washing, they were incubated with streptavidin-coupled peroxidase (1:500, Amersham Biosciences) for 30 min. After washing, labeling was performed using diaminobenzidine (Sigma), and spot intensity was quantified by densitometry (PhosphorImager, Amersham Biosciences). In some experiments, the antigen was incubated with MPB before blotting the sample on the nitrocellulose filter, blocking with PBS, 2% casein, and subsequent processing.

*Thiol Content of Surface-associated Env*—CEM cells (5 × $10^7$) were either treated using 1 mM bacitracin or mock-treated for 2 h. They were then incubated for 2 h at 37 °C in $CO_2$ atmosphere with gp120 (30 $\mu$g/500 $\mu$l) produced by baby hamster kidney 21 cells infected using VV vectors. Cells were washed and treated with $NaN_3$ (0.1%) to inhibit further surface remodeling (20). MPB was added to the cell pellet (0.3 mM, 30 min at 25 °C). Excess reagent was blocked using glutathione (0.6 mM, 10 min at 25 °C), and the remaining sulfhydryl groups in the system were quenched with iodoacetamide (1.2 mM, 10 min at 25 °C) (24). Cells were washed twice and incubated in acid buffer (MES/HCl 10 mM, NaCl 150 mM, pH 3.0) for 10 min to dissociate surface-bound Env as described previously (25, 26). The eluate was adjusted at pH 7.0 using NaOH, and Env was immunoprecipitated for 4 h at 4 °C using D7324 covalently coupled to CNBr-Sepharose CL4B (Amersham Biosciences) as described previously (27). Gp120 present in the original concentrated cell supernatant or in medium corresponding to supernatant containing gp120 subsequently to incubation with CEM cells (see above) was processed similarly. After elution from Sepharose-bound D7324 using 1% SDS, the purified envelope samples were dot-blotted onto nitrocellulose filter and processed as above to determine the corresponding thiol content. In parallel, dot-blot quantitation of the amount of gp120 present in the eluate after immunoprecipitation using D7324 was achieved by incubation with a pool of anti-HIV-1 polyclonal human IgG and staining using appropriate anti-IgG antibodies coupled to peroxidase and diaminobenzidine (19). Spot intensity was quantified by densitometry to enable gp120 quantitation by comparison with a standard curve obtained with a known reference batch of recombinant $gp120_{Lai}$. The protein content of the eluate was also assessed using a microquantitation assay (Pierce). Together, these assays allow MPB reactivity to be related to the amount of immunopurified Env to determine the thiol content per molecule of gp120.

*Labeling of Surface-associated PDI*—We examined the association of PDI with the outer cell membrane as described previously (28). CEM cells ($10^7$) were washed, resuspended in 0.5 ml of PBS containing 0.2 mM of MPB, and incubated for 30 min at room temperature to label the thiols of cell surface proteins. Quenching was performed using glutathione (0.4 mM, 10 min at 25 °C) and iodoacetamide (0.8 mM, 10 min at 25 °C). The cells were washed, sonicated in lysis buffer (ice-cold PBS containing 0.5% Triton X-100, 10 $\mu$M leupeptin, 2 mM phenylmethylsul-

fonyl fluoride, and 10 $\mu$M aprotinin), and incubated with streptavidin-agarose (Sigma) for 1 h at 4 °C in PBS containing 0.5% Triton X-100, 10 $\mu$M leupeptin, 2 mM phenylmethylsulfonyl fluoride, and 10 $\mu$M aprotinin. The beads were washed with PBS, and bound proteins eluted with SDS sample buffer, resolved on 10% SDS-PAGE, and transferred to nitrocellulose membrane. Anti-PDI polyclonal antibodies (SPA-890, 1/1000) and anti-rabbit antibodies coupled to peroxidase (1/2000, Sigma) were used for staining (15).

*Thiol Content of Surface-associated Proteins*—We assessed the thiol content associated with the native lymphocyte surface. Uninfected cells (3 × $10^6$) treated or not using PDI inhibitors were incubated with MPB (0.1 mM, 30 min at 25 °C), and excess reagent was quenched with glutathione and iodoacetamide as described above. After washing, cells were incubated with streptavidin peroxidase and then were incubated with the corresponding substrate before optical density reading. Alternatively, after cell lysis using PBS 1% SDS, the lysates were processed for dot-blot as described above. They were also analyzed by SDS-PAGE (10%), and after blotting, they were processed using streptavidin peroxidase as described above. The strips were scanned to quantitate MPB labeling.

## Env Treatment with β-Mercaptoethanol

Env was treated with β-mercaptoethanol (0–3%, 10 min at 25 °C) before dot-blotting, MPB labeling, and further processing as described above. For binding experiments, the sample was treated with β-mercaptoethanol and then with iodoacetamide (2.5:1 iodoacetamide:β-mercaptoethanol ratio, 30 min at 25 °C) to prevent further reoxidation of the thiol groups (29). After lyophilization to remove β-mercaptoethanol, the sample was processed for binding to CD4 and CXCR4 as described below.

## CD4 Binding Assay

The CD4 binding assay was performed as described previously (29). Env (100 ng) bound to D7324-coated microtiter plates was incubated with $^{125}I$ CD4 (2 × $10^4$ cpm/well) for 2 h. After washing, radioactivity was counted. Background binding was measured as the signal generated by similar plastic wells lacked Env. Unlabeled CD4 (150 nM) was used to determine nonspecific binding. To assess that the β-mercaptoethanol treatment did not modify the subsequent capacity of Env to bind antibody-coated wells, the binding of $^{125}I$ Env to microtiter plates was investigated for each β-mercaptoethanol concentration used as reported previously (29).

## CXCR4 Binding Assay

The CXCR4 binding assay was performed as described previously (19, 20). Env (2 $\mu$g/50 $\mu$l) was added to living CEM cells (3 × $10^6$) for 2 h at 37 °C in culture medium. Cells were then treated by 0.1% $NaN_3$ for 10 min and further incubated with $^{125}I$ SDF1-$\alpha$ (5 × $10^4$ cpm) for 1 h at 25 °C in buffer (RPMI 1640 medium, 10 mM HEPES, 5% bovine serum albumin, and 0.1% $NaN_3$). Cell-associated and free radioactivity were separated using the dibutylphtalate/bis(2-ethylhexyl)phtalate two-phase system. Unlabeled SDF1-$\alpha$ (200 nM) was used instead of Env to determine nonspecific binding.

## RESULTS

*Titration of Thiol*—To measure small changes in the thiol content of Env, a quantitative assay was developed using MPB, a membrane-impermeant compound coupled to biotin whose reaction with the thiols of proteins can be detected using streptavidin peroxidase. To establish the assay, we employed Tg as this glycoprotein exhibits a similar "cysteine to molecular weight" ratio to $gp120_{Lai}$ (17, 30). Preliminary experiments showed that (i) all the disulfide bonds of Tg are reduced using 1% β-mercaptoethanol and (ii) similar results were obtained when incubation with MPB was performed prior to or after immobilization of the protein onto nitrocellulose. Densitometry of reduced Tg (0.2–200 ng) blotted and processed using MPB produced a linear dose response between 2 and 40 ng. Taking into account its molecular mass (330 kDa) and Cys content (122 Cys residues/molecule) (30), we determined the thiol content for each protein sample. This provided a standard curve and allowed the development of an assay detecting thiols with an absolute sensitivity of 0.3 pmol (Fig. 1A) and a signal/background ratio of 50.
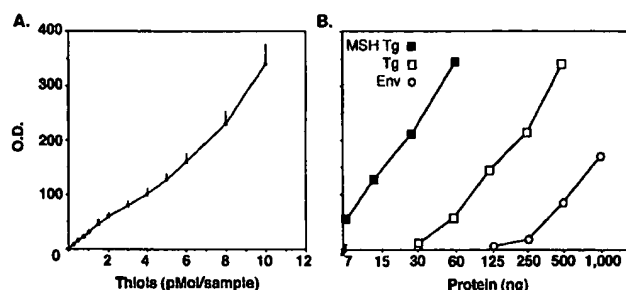
FIG. 1. **Thiol dosage.** *A*, standard curve. Samples containing increasing amounts (determined as described under "Results") of thiols associated with β-mercaptoethanol-treated Tg were blotted onto a nitrocellulose filter and stained using MPB. Labeling was performed using streptavidin-coupled peroxidase and diaminobenzidine. Spot intensity was quantified by densitometry and used to determine a standard curve ($n$ = 6, means ± S.D. are presented). *B*, MPB reactivity of Env and Tg. Increasing amounts of either gp120 (*Env*) or Tg treated (*MSH Tg*) or not treated (*Tg*) by β-mercaptoethanol were blotted onto a nitrocellulose filter and processed as described above ($n$ = 3, means ± S.D. are presented).
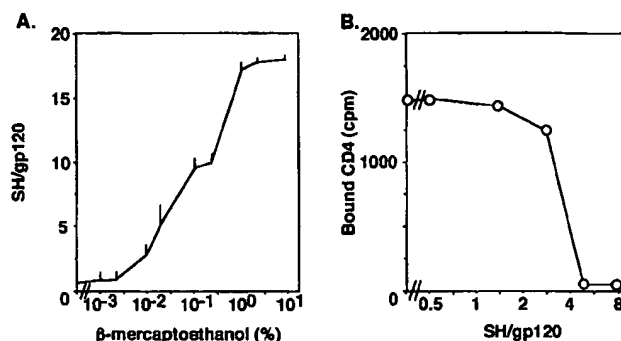


FIG. 2. **Thiol content and CD4 binding properties.** *A*, reduction of gp120 by β-mercaptoethanol. Gp120 was treated by increasing concentrations of β-mercaptoethanol. The thiol content of each sample (*SH/gp120*) was then determined using MPB. *B*, Env binding to CD4. Env samples (100 ng) presenting various thiol contents (SH/gp120) were incubated with ^{125}I CD4. Bound radioactivity was counted ($n$ = 2, means ± S.D. are presented).

Using these conditions, we verified that MPB reacted with native Tg in a dose-dependent manner and determined that its thiol content per molecule was 13 (Fig. 1*B*). Commercially available gp120$_{Lai}$ (Fig. 1*B*) and gp120$_{GB8}$ (data not shown) exhibited a low reactivity with MPB, which corresponded to 0.5–1 thiol/molecule. Thus, in native folded and secreted gp120, essentially all Cys residues are involved in disulfide bonds. The specificity and linearity of dosage observed for Tg were also observed with gp120 (Fig. 1*B*).

*Relationship between the Thiol Content of gp120 and Its Receptor Binding Capacity*—We then determined the relationship between the thiol content of Env and its capacity to bind lymphocyte receptors. Env was treated with various concentrations of β-mercaptoethanol, and the corresponding thiol content was determined (Fig. 2*A*). Alternatively, after reaction with β-mercaptoethanol, the sample was incubated with iodoacetamide, the reducing agent was removed by lyophilization, and the resulting Env was assayed for CD4 binding as described previously (29). We observed that reduction of one disulfide bond was tolerated but the reduction of more than one prevented CD4 binding (Fig. 2*B*).

β-Mercaptoethanol-treated Env was tested for its CXCR4 binding capacity (19, 20). Env and the natural CXCR4 ligand, SDF1-α, share a binding site on CXCR4 that allows Env binding to be measured indirectly through competition with SDF1-α (31), although low receptor affinity limits maximum inhibition using Env to ~50% (19, 32, 33). The reduction of a single disulfide bond impaired the capacity of gp120 to compete with ^{125}I SDF1-α binding at the lymphocyte surface (Fig. 3*A*). The treatment of the cell surface by bacitracin did not block SDF1-α binding inhibition by Env (Fig. 3*B*), indicating that Env binding to CXCR4 did not require PDI activity. As a control, ^{125}I SDF1 binding was inhibited by SPC3 (10⁻⁵ M), a V3-derived peptide that interacts with the binding site of Env on CXCR4 (19, 20, 33).

*Development of Env Disulfide Bonds during the Course of Membrane Fusion*—MPB was first used to biochemically demonstrate the presence of PDI on the CEM cell surface. Cells were incubated with MPB or a mock. Samples were washed, lysed, and incubated with streptavidin-agarose before the eluates were separated by SDS-PAGE and blotted with anti-PDI antibodies (28). A single band was detected migrating with the apparent molecular mass of PDI that was significantly enriched by the MPB labeling when compared with the mock-treated sample (Fig. 4*A*). Thus, PDI on the CEM cell membrane is accessible to the exogenous reagent. We next examined the
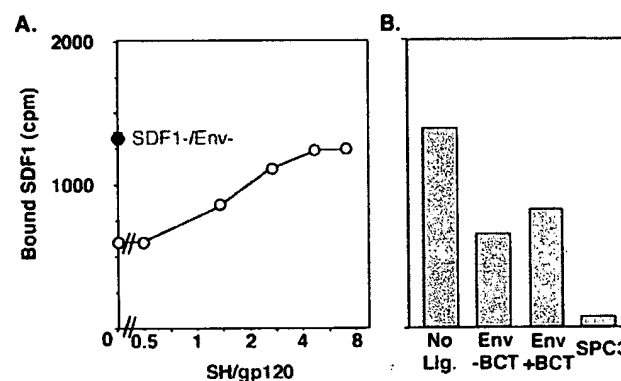


FIG. 3. **Thiol content and CXCR4 binding properties.** *A*, Env binding to CXCR4. Living CEM cells were incubated at 37 °C (i) with Env samples (1 μg/50 μl) presenting various thiol contents (SH/gp120); (ii) with unlabeled SDF1-α (200 nM) (it determines the background signal level); or (iii) in control conditions (*SDF1-/Env-*, maximum signal). [125]I SDF1-α was then added for 1 h in buffer supplemented with 0.1% NaN₃, and the radioactivity associated with the cell pellet was counted ($n$ = 2, means ± S.D. are presented). *B*, effect of bacitracin. CEM cells were preincubated (+*BCT*) or not (−*BCT*) with bacitracin for 2 h at 37 °C. Gp120 (3 μg) (*Env*) was then added. Alternatively, cells were incubated with either unlabeled SDF1-α (200 nM) (it determines the background signal level) or SPC3 (10⁻⁵ M) (*SPC3*), an anti-HIV V3-derived peptide that binds the Env binding site on CXCR4. Labeled SDF1-α was then added, and cells were processed as described above. Maximum ^{125}I SDF1-α binding inhibition was determined in the absence of unlabeled CXCR4 ligands (*No lig.*) ($n$ = 2).

capacity of bacitracin to alter the reductive activity associated on the CEM cell surface. Treatment with 1 mM bacitracin reduced MPB reactivity by 50% (Fig. 4*B*). This result is consistent with inhibition of surface PDI and is similar to the reduction in the thiol pool of the cell surface obtained using anti-PDI antisense phosphorothioates (34). For specificity, cell preincubation with thiol reagents was shown to dramatically reduce MPB reactivity.

We then addressed the thiol content of Env after its interaction with either CD4 or both CD4 and CXCR4. Gp120 was incubated with CEM cells as follows: (i) cells and gp120 incubated without prior incubation with SDF1-α and sCD4 (SDF⁻/CD4⁻); (ii) cells preincubated with SDF1-α (2 × 10⁻⁶ M to block CXCR4) before incubation with gp120 (SDF⁺/CD4⁻); and (iii) cells preincubated with SDF1-α and Env preincubated with sCD4 (0.5 × 10⁻⁶ M) before the addition of gp120 to cells (SDF⁺/CD4⁺). Concentrations of sCD4 and SDF1-α were saturating (19, 27). After incubation with Env, thiol labeling of the cell
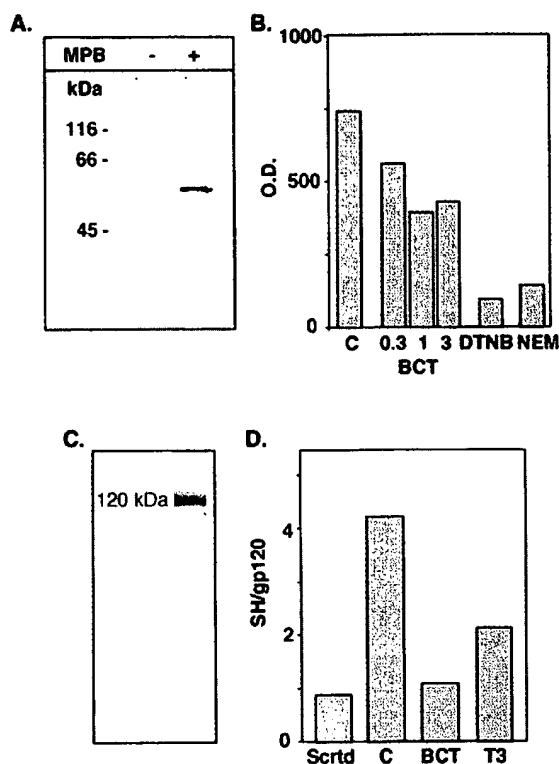
FIG. 4. *A*, association of PDI with the CEM cell surface. CEM cells were incubated with MPB (+) or not (−). Cell lysates were incubated with streptavidin-agarose. Proteins associated with the gel were resolved on SDS-PAGE and Western blotted using anti-PDI antibodies. *B*, MPB labeling of the lymphocyte surface. CEM cells were treated with either bacitracin (*BCT*) (0.3, 1, and 3 mM) or thiol reagents (1 mM DTNB; 1 mM *n*-ethylmaleimide (*NEM*)). Cells maintained in control conditions were also studied (*C*). After washing, cells were incubated with MPB and then with streptavidin peroxidase. The activity associated with the cell surface was assessed using orthophenylene diamine (*n* = 4, a representative experiment is shown). *C*, immunoprecipitation of gp120 associated with the lymphocyte surface. After incubation of CEM cells with gp120 and washing, the envelope protein was acid-dissociated from the cell surface, purified using Sepharose-coupled D7324 antibody, and analyzed by SDS-PAGE and protein staining. *D*, inhibition of gp120$_{89-6}$ disulfide bond cleavage following lymphocyte binding. CEM cells were treated using T3 (*T3*) (100 μM), bacitracin (*BCT*) (1 mM) or mock-treated (*C*). They were then incubated with gp120$_{89-6}$ before MPB labeling. Env was then acid-dissociated and immunoprecipitated before thiol content assessment (*SH/gp120*). The thiol content of secreted gp120$_{89-6}$ (*Scrtd*) was determined in parallel.

surface components was performed using MPB. In samples described in (i) and (ii), labeled Env was recovered from the lymphocyte surface by an acid wash (25, 26) and captured using the C-terminal peptide antibody D7324 coupled to Sepharose, resulting in the recovery of pure intact gp120 (Fig. 4*C*). In sample described in (iii), the cell medium containing Env that did not bind the cell surface was similarly incubated with D7324-Sepharose.

The thiol content of the immunopurified samples was determined as before, and the amount of gp120 was assessed to determine the thiol/gp120 ratio. The thiol content of gp120 associated with the lymphocyte surface in conditions where both CD4 and CXCR4 were accessible was found to be 4-fold higher than that of either immunopurified Env from the original supernatant or of gp120 present in the cell supernatant of the SDF$^+$/CD4$^+$ sample (Table I). In the SDF$^+$/CD4$^-$ sample, there was no significant change in the thiol content of cell-associated Env when compared with gp120 in the original supernatant.

The significance of lymphocyte surface PDI activity on the redox changes observed for lymphocyte-associated Env was

addressed using 1 mM bacitracin. The inhibitor prevented the increase of the thiol content observed in the SDF$^-$/CD4$^-$ condition (Table I), whereas it neither interfered with Env binding to CD4 (9) and CXCR4 (see above) nor the reaction of MPB with thiols of proteins (data not shown).

Bacitracin reacts with the redox active CXXC sequences of PDI to block the catalyst (10, 13) and is a specific and potent PDI inhibitor (34). T3 is a weaker inhibitor that exerts its activity through binding the PDI/substrate interaction domain (18). To independently confirm both the PDI dependence of Env reduction and its physiopathological relevance, we did experiments using 200 μM T3, a dose that inhibits PDI activity (18), and gp120$_{89-6}$, an envelope derived from a dual-tropic primary isolate (22). We observed that Env$_{89-6}$ was reduced upon interaction with the cell surface and that bacitracin and, to a lesser extent, T3 inhibited Env$_{89-6}$ reduction (Fig. 4*D*). We conclude that the thiol content of Env increases after interaction with a lymphocyte surface competent for CXCR4 binding and that this change depends upon a surface PDI activity.

To confirm this data using a biological system mimicking the HIV/lymphocyte interaction, we made use of Env$_{LAI}$ expression on the surface of CD4$^+$ human lymphocytes. In this system, cell densities of greater than 10$^6$ cells/ml permit syncytium formation, whereas those below 10$^5$ cells/ml do not (9, 23). MPB labeling was done on the surface of the same number of Env-expressing cells cultured at either of the two densities, and cell surface Env was isolated as before. The thiol content and the amount of purified gp120 were assessed to determine the thiol/gp120 ratio. We found that Env associated with the surface of the dense culture (syncytium forming) had an MPB reactivity that was 3-fold higher than that associated with the surface of cells maintained at low cell density (Table I). Because various PDI inhibitors prevent syncytium spread in this system (9), we conclude that changes in the thiol content of lymphocyte surface-associated Env are obligatory for fusion.

## DISCUSSION

HIV Env is an unusually highly disulfide-bonded molecule with 9 of a total of 10 disulfide bonds occurring within the outer membrane domain of gp120 (17). Gp120 is functionally complex as it interacts with at least three ligand surfaces to trigger HIV entry: 1) CD4, the primary receptor, 2) CCR, the secondary receptor, and 3) gp41, the fusogenic partner (3, 6). In addition, in the course of fusion, this heavily glycosylated protein (35) must be sufficiently pliable to not sterically inhibit the confor-
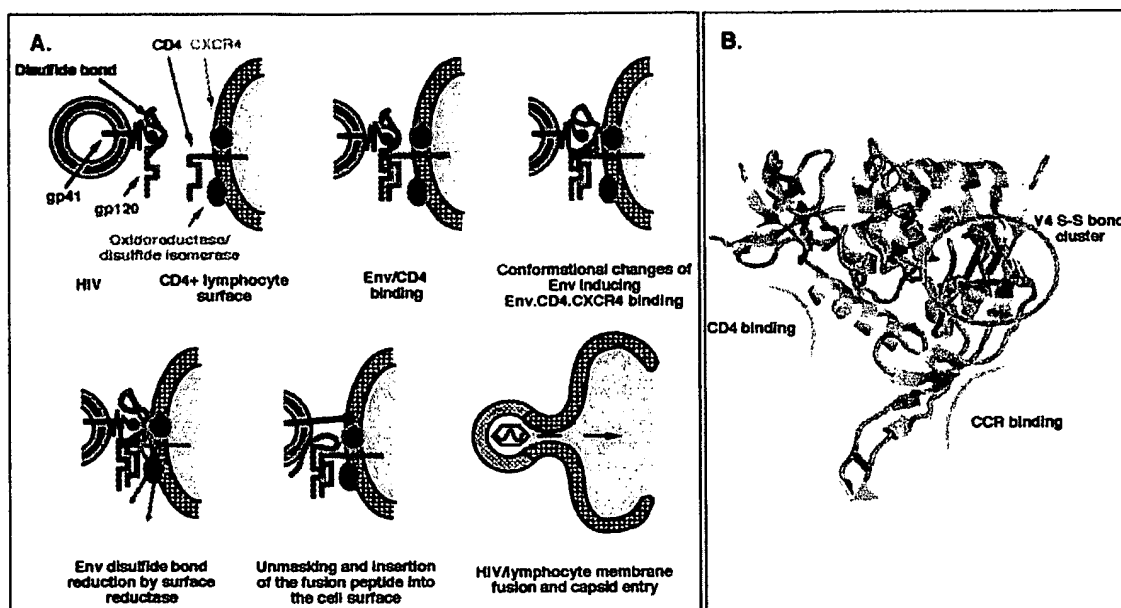
FIG. 5. *A*, HIV/lymphocyte interaction. The main steps of the HIV/lymphocyte interaction process are shown. Based on the data presented here, the step where a reductase activity belonging to the PDI family takes place is indicated. *B*, Gp120 structure. The image was rendered from the coordinates of the three-dimensional structure of gp120 (16) using RASMOL (40). The molecular surfaces involved in binding the primary receptor, CD4, and the secondary receptor, CXCR4, are shown. Cys residues are shown in *cyan*, and disulfide linkages are shown in *red*. Of particular note is the cluster of disulfide bonds that occurs at the base of variable loop 4 (as indicated) and in close proximity to the CCR binding site. Disulfide bond reduction at this location would be consistent with the increase in thiol dosage documented in the text.

mational changes required for virus-cell fusion (2, 5). Here, we demonstrate that the later stages of the process leading to fusion are enabled by the reduction of disulfide bonds of Env by a lymphocyte surface-associated reductase activity.

Two articles suggested that changes in the redox status of the disulfides of Env post-synthesis may occur in relation to HIV entry into the target cell (9, 10). Ryser *et al.* (10) reported that HIV infection of human lymphocytes was markedly inhibited by 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), bacitracin, and antibodies directed against PDI. More recently, we showed that these inhibitors altered the HIV receptor-dependent gp41-mediated fusion process *per se* at a post-CD4 binding step (9). Neither study, however, provided proof that the inhibition of PDI impaired thiol/disulfide changes within Env that could be correlated with abrogation of fusion. Here, using systems that address the interaction of Env with an authentic lymphocyte surface in the context of the various catalytic activities that lead to fusion (7-9), we clarified this issue and add three important new dimensions to previous studies: (i) the development of a sensitive assay for the quantitation of discrete changes in the thiol content of proteins, (ii) identification of the minimum disulfide bond integrity required for Env functional binding, and (iii) the stage of fusion at which thiol emergence occurs.

The relationship between the integrity of the disulfides of gp120 and its capacity to interact with viral receptors was examined initially. We found that a population of mature gp120 species exhibiting on average one reduced bond was capable of CD4 binding, whereas CXCR4 binding did not tolerate any alteration in the redox state of Env. We also found that bacitracin had no direct effect on Env binding to CXCR4. These data indicate that the presence of thiols on Env until CXCR4 binding occurs is inconsistent with the development of the process leading to fusion.

We then examined the redox state of gp120 as part of its interaction with a CD4$^+$ human lymphocyte surface. Firstly, we found that the thiol content of gp120 after interaction with a native cell surface displayed about 4 thiols per molecule

compared with <1 thiol in the original population of soluble antigen. This observation was done both for a gp120 derived from a laboratory-adapted HIV strain or from a dual-tropic primary isolate. Using high concentrations of SDF1-$\alpha$ and/or sCD4, we obtained conditions that specifically impaired Env binding to either one or both surface antigens (19, 27). A comparison of the data obtained when cells were preincubated or not with SDF1-$\alpha$ showed that changes in the redox status of gp120 required the availability of the binding site for Env on surface CXCR4. Secondly, Env associated with the surface of the dense (syncytium-forming) culture infected using VV9-1 had a thiol content that was 3-fold higher than that associated with the surface of cells maintained at low cell density. These results and our previous report that PDI inhibition prevented syncytium spreading but not CD4 binding (9) allow us to conclude that changes in the thiol content of Env are a requirement of the fusion reaction.

We previously reported that PDI is clustered at the lymphocyte surface in the vicinity of CD4-enriched regions and that some colocalization occurs (9). We showed here that PDI is labeled on the cell surface by the impermeant thiol probe MPB, further demonstrating its accessibility on the outer membrane of the lymphocyte cell line. As both PDI-specific inhibitors and anti-PDI antibodies inhibit HIV/lymphocyte fusion post-CD4 binding (9), it seems probable that a member of this class of catalysts may be the mediator of Env reduction. A direct evidence supports this conclusion because the use of PDI inhibitors prevented the increase in thiol content of lymphocyte surface-associated gp120 in the SDF$^-$/CD4$^-$ sample. Our data also show that the oxidizing nature of the cell surface still permits the persistence of thiols resulting from disulfides reduction of proteins, in agreement with a recent report (36).

Because the increase in the thiol content of monomeric soluble gp120 after its interaction with the cell surface was similar to that observed for oligomeric gp120 associated with syncytia, we conclude that changes in redox state do not depend on Env oligomeric status or on the presence of gp41. Our capacity to detect an increase in the thiol content of Env associated with

syncytia is consistent with a report demonstrating that the surface of fusing cells is heavily enriched in fusion-competent Env species (37).

Our study shows that a lymphocyte surface-associated PDI-related reductase activity assists the cleavage of, on average, two disulfide bonds within Env subsequent to its interaction with CXCR4 and that it is obligatory for fusion (Fig. 5A). We speculate that the Env reduction process and the disruption of the Env disulfide network it promotes assist post-receptor binding Env conformational changes and are necessary for acquisition of conformation competent to trigger membrane fusion (4, 6). Indeed, if the surface-associated form of PDI can act as a redox-driven chaperone to unfold proteins after disulfide bond reduction, as has been shown for its endoplasmic reticulum counterpart (38), it may directly catalyze the conformational changes occurring within Env required to ultimately unmask the gp41 fusion peptide. Recently, the reduction of the second domain of CD4 was reported to be an obligatory step in CD4-dependent fusion (39). Our results raise the possibility that redox changes observed within CD4 may be the consequence of thiol/disulfide interchanges occurring within the CD4·CXCR4·Env complex after Env reduction by PDI.

Our data do not address which disulfide bonds undergo reduction post-CXCR4 binding, but we note that a cluster of bonds occurs in gp120 at the base of the V4 region important for Env bioactivity (16). Disulfide bond reduction at this location could reduce a number of bonds at one time and could bring about considerable conformational change (Fig. 5B).

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